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Limited Expansion of Virus-Specific CD8 T Cells in the Aged Environment

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Abstract

The mechanisms responsible for the diminished immune response seen with aging are unclear. In this study, we investigate the contributions of alterations in the lymphoid microenvironment to this decrease. Using adoptive transfer of virus-specific transgenic CD8 T cells, we demonstrate that the aged environment inhibits the clonal expansion of specific CD8 T cells from young mice during virus infection. Transferred specific CD8 T cells from young mice demonstrated a response reflecting the CD8 T cell response of the intact aged host: the CD8 T cells expand more slowly and have a decreased maximal expansion in an aged compared to a young environment. While isolated DCs (MHC II^+ $CD11c^+$) of aged mice maintain their ability to support CD8 T cell Ag-specific expansion *in vitro*, splenocytes demonstrated an age-associated decrease in this ability. Since the percentages of various populations of DCs in splenocytes demonstrate no significant alteration with age, this diminished APC activity of splenocytes of aged mice may reflect inhibitory activity of other cell populations. The results of this study demonstrate that elements of the aged environment play an important role in the alteration of T cell response to virus infection in the aged.

Keywords

T Cells; Viral; Dendritic Cells; Transgenic Mice; Aging

1. Introduction

One of the hallmarks of aging is the overall decline in immunocompetence, which leads to a variety of decrements in immune function, including the T cell response to virus infection (Kapasi et al., 2002; Gardner and Murasko, 2002). T cell function in both aged mice and humans has been characterized by decreased proliferation and altered cytokine production (Gardner and Murasko, 2002). The decline in CD8 T cell immunity with aging has been widely reported, although the mechanisms are still largely unknown. Both intrinsic and extrinsic factors are

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considered to affect CD8 T cell response with aging (Murasko and Jiang, 2005; Linton et al., 2005). In a previous study, we reported that upon stimulation of CD8 T cells with the T cell mitogen Con A, a larger percentage of CD8 T cells of aged mice start proliferation later and stop sooner compared with CD8 T cells of young mice (Jiang et al., 2007), indicating an intrinsic alteration in CD8 T cells of aged mice. While this and other studies suggest a general defect in T cells of aged mice, some reports demonstrate limited changes in T cell responses with age. For example, one study showed that age-associated alterations in naïve CD8 T cell function are not found after primary Ag stimulation, but may become apparent upon re-stimulation (Li et al., 2002). While another study indicated anti-tumor immunity mediated by CD8 T cells of aged mice is not impaired (Norian et al., 2004), suggesting minimal intrinsic deficiency in anti-tumor functions of CD8 T cells with aging.

Many studies have been performed in vitro to assess T cell function and other aged-related immunological alterations. However, T cell function is influenced by cellular and soluble factors of the lymphoid environment in vivo, such as cytokines, APCs etc. Mittler et al (Mitter et al., 2004a and 2004b) reported that the aged lymphoid microenvironment inhibits memory, but not naïve, CD4 T cell response. Dendritic cells (DCs) are one of the components of the environment which influence T cell responses. To what extent alterations in APC function leads to the decrease of T cell responsiveness with aging remains unanswered. Several studies have described a reduced ability of aged unfractionated APCs or macrophages to stimulate T cell effector function (Plowden et al., 2004; Donnini et al., 2002; Beharka et al., 1997; Sprecher et al., 1990). However, human monocyte-derived DCs from aged donors appear phenotypically similar to those from young donors and have been reported to be equally capable of stimulating Ag-specific T cell responses in vitro (Lung et al., 2000).

The impact of the aged host environment on specific CD8 T cell response to virus infection has received limited attention (Tesar et al., 2006). Thus, it is important to explore whether any aged-related changes in the lymphoid microenvironment might affect the specific CD8 T cell response to viral Ag-stimulation. If the aged environment significantly impacts specific CD8 T cell response, strategies for developing more effective vaccines to prevent infectious diseases in the aged will need to target the environment in addition to T cells.

In the present study, we found that the response of specific CD8 T cells of young mice to virus infection is impaired in the aged environment. Using an adoptive transfer approach with two different viruses, we observed that the aged environment, regardless of genetic background of mice, substantially inhibits clonal expansion of specific CD8 T cells and IFN-γ production during virus infection. These data suggest that alterations in the aged environment might play a critical role in the decreased specific CD8 T cell immunity to virus infection with aging.

2. Materials and Methods

2.1. Mice

Four-month- and 22-month-old wild type BALB/c and C57BL/6 (B6) mice were purchased from the NIA at Harlan Sprague Dawley (Indianapolis, IN). Six to 8 week old P14 ($GP_{33–41}$ TCR-Tg) mice (Ashton-Rickardt et al., 1994) were purchased from Taconic Farms (Hudson, NY), and 6 to 8 week old BALB/c ByJ Cl.1 Thy1.1, Clone-4 ($HA_{518–526}$ TCR-Tg) mice (Kreuwel et al., 2001) were obtained from Jackson la boratory (Bar Harbor, Maine). All mice were maintained in AAALAC-approved barrier facilities at Drexel University (Philadelphia, PA). Mice were allowed to acclimate for at least one week in our animal facilities prior to use. Mice exhibiting enlarged spleens or tumors were eliminated from this study. All experiments involving mice were conducted with the approval of the Institutional Animal Care and Use Committee (IACUC) at Drexel University.

2.2. Virus infections

Influenza A- Puerto Rico/8/34 (PR8; H1N1) viruses were propagated in specific-pathogen-free eggs and stored at −80 °C for subsequent use. Mice were infected intravenously (i.v.) with 300 μl of sterile saline containing 300 Hemagglutination units (HAU)[3] of PR8. In some experiments, mice were infected intraperitoneally (i.p.) with 2×10^6 PFU of lymphocytic choriomeningitis virus (LCMV) Armstrong strain (Jiang et al., 2003) in 300 μl of sterile saline.

2.3. Cell preparation

Mice were sacrificed by CO_2 asphyxiation followed by cervical dislocation, and spleens were aseptically removed. Lymphocytes were isolated using 0.83% ammonium chloride from young and aged mice. Mononuclear cells were resuspended in RPMI-1640 containing 10% FBS, L-glutamine, 2-β-mercaptoethanol (Sigma-Aldrich), and gentamycin (EL4-media), and aliquots evaluated by surface staining, tetramer binding, and intracellular cytokine staining. Purification of CD8 T cells and DCs was performed by MACS using CD8a and CD11c Microbeads, respectively (Miltenyi Biotec, Bergisch Gladbach, Germany). The phenotype of the cells was determined by flow cytometry before and after purification.

2.4. Adoptive transfer of splenocytes from TCR Tg mice

Splenocytes were isolated from TCR Tg mice (Clone-4 mice or P14 mice) and labeled with 5 μM CFSE (Molecular Probes, Eugene, OR) in PBS, then quenched with 100% FBS. Cells were then washed with RPMI 1640/10% FBS and resuspended in PBS. Different doses of splenocytes from Clone-4 mice ($Thy1.1^+$) or CFSE labeled splenocytes from P14 mice were adoptively transferred i.v. into BALB/c or B6 recipient mice, respectively. One day after transfer, young and aged recipients were infected with PR8 or LCMV as described above.

2.5. Priming of specific CD8 T cells in vitro

1×10^5 purified CFSE labeled-TCR-Tg cells (P14 or Clone-4 cells) were cultured with 1μM peptide ($GP_{33–41}$ or $HA_{518–526}$, respectively) and 5×10^5 splenocytes or 1×10^4 enriched DCs of wt young or aged mice in RPMI-1640 media (containing 10% FBS, L-glutamine, 2-β-mercaptoethanol, and gentamycin). Twenty-four to 72 h after incubation with or without peptide at 37°C, the cells were harvested and stained with antibodies to examine the proliferation and expansion of specific TCR-Tg cells by flow cytometry.

2.6. Flow cytometry

2×10^6 splenocytes were stained for surface markers using mAbs. Anti-CD8, CD44, CD11b, CD40, CD86, B220, Thy1.1, MHC I-A^b, and MHC I-A^d Abs were purchased from BD PharMingen (San Diego, CA), and anti-CD209, CD11c, and CD80 mAbs were obtained from eBioscience (San Diego, CA). H-$2K^d$ $HA_{518–526}$, and H-$2D^bNP_{366–374}$ tetramers were obtained from NIAID MHC Tetramer Core Faciliy (Atlanta, GA). Intracellular IFN-γ staining was performed using anti-IFN-γ mAb (BD PharMingen) with the Cytofix/Cytoperm kit (BD Pharmingen). In this procedure, splenocytes were cultured with $GP_{33–41}$(KAVYNFATC, GP), $HA_{518–526}$ (IYSTVASSL, HA), or $NP_{366–374}$ (ASNENMETM, NP) peptide (SynPep, Dublin, CA) for 5 h in the presence of GogiStop. The cells were stained for surface markers, including tetramer, then fixed, permeabilized and stained for IFN-γ. Flow cytometry was performed with a FACS CanTo (Becton Dickinson, San Jose, CA), and data was analyzed with FlowJo software (Tree Star, Inc., San Carlos, CA).

2.7. Statistical analysis

All statistical analyses were performed using Student's t test. Significant differences were determined at the level of $p < 0.05$. Results are expressed as mean ± SD.

3. Results

3.1. Decreased Ag-specific CD8 T cell response of aged mice after influenza virus infection

We had previously demonstrated that aged B6 mice demonstrate both a decreased and delayed CD8 T cell response to primary influenza infection as assessed by binding of virus-specific tetramers ($NP_{366–374}$) (Po et al., 2002). In order to assess the role of the environment, we wanted to utilize CD8 T cells from mice transgenic (Tg) for an influenza-specific TCR. Although no such Tg mouse is available commercially on the B6 background, one is available on the BALB/c background (Clone-4). Before adoptive transfer of Clone-4 influenza-specific TCR Tg CD8 T cells, we wanted to ascertain that a similar age-associated decrease in CD8 T cell response to influenza infection occurred in BALB/c mice. We, therefore, infected both B6 and BALB/c mice with influenza virus (PR8) i.v. The H-$2D^b$-NP and H-$2K^d$-HA tetramers were utilized to measure the specific CD8 T cell response in B6 and BALB/c mice, respectively. As shown in Fig. 1A, the percentage of NP^+ specific CD8 T cells in spleens were significantly lower in aged than in young B6 mice on Day 7 after infection (Young vs aged: 4.55 ± 0.72% vs 0.64 ± 0.06% of CD8 T cells, $p < 0.05$). Consistent with this observation in B6 mice, the percentage of HA^+ specific CD8 T cells detected in spleens of BALB/c mice was also significantly lower in aged than in young mice 7 days after infection (Fig. 1B: Young vs aged: 1.41 ± 0.14% vs 0.25 ± 0.05% of CD8 T cells, $p < 0.05$), although the magnitude of the response was consistently lower in BALB/c mice. Further, the peak expansion of the specific CD8 T cells generated occurred on Day 7 and Day 10 in young and aged mice, respectively, of both strains (B6: ref Po et al, 2002; BALB/c: Day 10: Young vs Aged: 0.5 ± 0.1% vs 0.6 ± 0.1% of CD8 T cells, $p > 0.05$). These data confirm our previous results in B6 mice (Po et al., 2002) and extend the observation to BALB/c mice that the CD8 T cell responses to primary influenza infection is both decreased and delayed with aging.

In addition to assessment of virus-specific response with tetramer staining, the presence of intracellular IFN-γ can also be used to determine the magnitude of CD8 T cells response to the virus-specific epitope (Serbina and Pamer, 2003; Appay and Rowland-Jones, 2002). Examination of intracellular IFN-γ^+ CD8 T cells of B6 mice on Day 7 after infection revealed that the percentages of IFN-γ^+ CD8 T cells of both young and aged mice reflected the percentage of CD8 T cells positive for the influenza-specific TCR (NP), with the frequency being significantly higher in young than in aged mice (Fig. 1C: Young vs aged: 4.11 ± 0.19% vs 0.53 ± 0.04% of CD8 T cells, $p < 0.05$). We initially assumed that due to the similarity of the percentage of IFN-γ^+ and NP^+ CD8 T cells, that all NP^+ T cells would also be IFN-γ^+. We, therefore, gated on NP^+ CD8 T cells and determined the percent positive for intracellular IFN-γ. As shown in Table 1, in young mice about 60% of the NP^+ CD8 T cells were also IFN-γ^+, while in aged mice only 36% of the NP^+ CD8 T cells on Day 7 and 47% at the peak of response (Day 10) were IFN-γ^+, suggesting that fewer of the virus specific cells were functional in aged mice. We then gated on IFN-γ^+ CD8 T cells and determined the percent specific for virus by NP tetramer binding. Ninety percent of the IFN-γ^+ cells of young mice were specific for NP (i.e. NP^+, IFN-γ^+). Since the percentage of NP^+ $CD8^+$ and IFN-γ^+ $CD8^+$ T cells of aged mice were very similar (eg. 1.9% for each on Day 10), we assumed that most if not all of the IFN-γ^+ CD8 T cells would be NP^+ as well. However, we observed that only 48% of the IFN-γ^+ CD8 T cells of aged mice bound NP tetramer on Day 7 after infection, increasing to only 65% on Day 10. These results demonstrate a delay in the response of aged mice not only in number but also in functional activity. Further, these data suggested that functional (as determined by IFN-γ) CD8 T cells of aged mice either were reactive to another epitope of influenza or were non-specific (“ bystander activation”). These results indicate that not only are there fewer virus-specific CD8 T cells in aged mice but also that a smaller proportion of these virus-specific CD8 T cells generate IFN-γ in aged than in young mice, and that more bystander CD8 T cells may be activated in aged than in young mice during virus infection.

3.2. Aged environment impairs specific CD8 T cell response to virus infections

To examine if the aged environment has any effect on the specific CD8 T cell response, we employed an adoptive transfer model using Thy1.1$^+$ Clone-4 TCR Tg cells that are specific to the H-2K^d-restricted HA epitope of PR8 (Kreuwel et al., 2001). 2–4 × 10^6 splencytes from young Clone-4 TCR-Tg mice (Thy1.1$^+$) were adoptively transferred into young and aged (Thy1.2$^+$) BALB/c mice. Recipient mice were then infected i.v. with PR8. The donor CD8 T cells in the spleen were examined for expansion and functional activity. Tetramer staining demonstrated decreased expansion of the HA$^+$ TCR-Tg T cells after virus infection in aged compared with young recipients in both percentage (Fig. 2A: Young vs aged: 23.1 ± 1.1% vs 11.5 ± 3.3%, $p < 0.05$) and absolute numbers of donor CD8 T cells (Fig. 2B: Young vs aged: 1.2 × 10^6 ± 0.1 vs 0.4 ×10^6 ± 0.2, $p < 0.05$). To further analyze the Ag-specific response, we measured the functional activity of virus-specific donor CD8 T cell in young and aged hosts by intracellular IFN-γ staining. On Day 3 after infection, we observed both a decreased percentage and number of IFN-γ$^+$Thy1.1$^+$ cells of CD8 T cells in the spleen of aged compared to young hosts (Fig. 2C: Percentage of total CD8 T cells: Young vs aged: 22.8 ± 1.1% vs 10.1 ± 3.1%, $p < 0.05$; Fig. 2D, Absolute number: Young vs aged: 10.5 ×10^5 ± 0.8 vs 3.6 ×10^5 ± 1, $p < 0.05$). Interestingly, on Day 7 after infection, the Tg CD8 T cell response in the young environment had contracted and no significant difference in Tg CD8 T cell numbers was observed between young and aged recipient mice (Young vs aged: 3.5 ×10^5 ± 1.2 vs 3.2 ×10^5 ±1.3, $p > 0.05$), while the level of intracellular IFNγ generated by Tg T cells in aged was significantly lower than in young recipients (mean fluorescence of intensity of IFNγ: Young vs aged: 1.5 ×10^4 ± 0.1 vs 0.9 ×10^4 ± 0.1, $p < 0.05$).

Next, we examined whether or not this limited expansion of young virus-specific CD8 T cells was restricted to the BALB/c background. Although we did not have access to influenza specific TCR Tg mice on the B6 background, LCMV TCR Tg mice were available, i.e., P14 mice. Before transfer, the splenocytes of young P14 mice were labeled with CFSE, a vital fluorescent dye that is equally partitioned into daughter cells, allowing visualization of cell division (Lyons et al., 2000). 2–4 × 10^6 CFSE-labeled splenocytes of young P14 TCR-Tg mice were adoptively transferred into young and aged B6 mice. The recipients were infected with LCMV (Armstrong strain) and the D^b-GP specific CD8 T cell response in the spleen was examined using D^b-GP specific tetramer binding and intracellular IFN-γ production at different times after infection. No expansion of the P14 cells was observed on Days 1 and 2 after infection in either age group (data not shown). On Day 3 post-infection, both the percentage and number of D^b-GP tetramer$^+$ CD8 T cells were significantly increased in young compared to aged recipients (Fig. 3A & 3B: Young vs aged: Percentage of total CD8 T cells: 5.48 ± 0.21% vs 1.2 ±0.27 %, $p < 0.01$; Fig. 3C: Young vs aged: Absolute number: 16.1 ×10^4 ± 0.7 vs 3.5 ×10^4 ± 1.2, $p < 0.01$). An interesting observation is that the percentage of adoptively transferred cells that did not proliferate (retained CFSE) was both minimal and fairly comparable in young and aged recipients (Fig. 3A: Young vs aged: 0.26% vs 0.4%). Consistent with the expansion of GP tetramer$^+$ CD8 T cells, considerable expansion of IFN-γ$^+$ CD8 T cells was observed in young, but not aged, recipients (Fig. 3D & 3E: Young vs aged: Percentage of total CD8 T cells: 7.88 ± 1.05% vs 1.67 ± 0.45%, $p < 0.05$; Fig. 3F: Young vs aged: Absolute number: 30.7 × 10^4 ± 2.4 vs 5.5 × 10^4 ± 1.8, $p < 0.01$). These results demonstrate that the aged environment impairs specific CD8 T cell response to virus infection, and suggest that the impairment is not virus or mouse background specific.

It has been reported that adoptive transfer of 10^6 TCR transgenic T cells is supraphysiological and can alter various parameters of the subsequent response (Badovinac et al., 2007; Obar et al., 2008; Marzo et al, 2005). To determine if transfer of a smaller number of cells will affect the results obtained above, we adoptively transferred 5×10^3 splenocytes of Clone-4 cells (Thy1.1$^+$). On Day 4 after infection with PR8, the number of donor CD8 T cells in the spleens

of young mice was significantly higher than that of aged mice, as identified by HA tetramer$^+$Thy1.1$^+$ staining (Fig. 4A: Percentage of total CD8 T cells: Young vs aged: 1.6 ± 0.1% vs 0.9 ± 0.1%, $p < 0.05$; Fig. 4B: Absolute number: Young vs aged: 19 ×10^4 ± 1.8 vs 4.2 ×10^4 ± 0.9, $p < 0.05$), and by intracellular IFN-γ staining after in vitro stimulation with $HA_{518–526}$ peptide for 5 h (Fig. 4C: Percentage of total CD8 T cells: Young vs aged: 1.65 ± 0.1% vs 0.88 ± 0.09%, $p < 0.05$; Absolute number: Fig. 4D: 15.5 ×10^4 ± 1.6 vs 3.1 ×10^4 ± 0.7, $p < 0.05$). In contrast to the previous experiment (Fig. 2) in which a larger number of Tg CD8 T cells was transferred, the expansion of specific Tg CD8 T cells was minimal on Day 3 after infection (data not shown), and on Day 7 after infection the expansion of Tg CD8 T cells was greater in aged mice than it was on Day 4, but was still significantly decreased in aged compared to young mice (HA tetramer$^+$Thy1.1$^+$: Young vs aged: 18 ×10^4 ± 1.3 vs 6.8 ×10^4 ± 1.1, $p < 0.05$). These data demonstrate that the aged environment limits the expansion of adoptively transferred specific Tg CD8 T cells regardless of the number transferred.

3.3. Contribution of number and Ag presenting function of Ag-specific CD8 T cells in aged mice

To examine whether the limited expansion of TCR Tg CD8 T cells in an aged environment was due to the altered ability of splenocytes to present antigens to CD8 T cells, we incubated purified CFSE-labeled Clone-4 TCR-Tg T cells with HA peptide and splenocytes from young or aged wt BALB/c mice. When the Clone-4 cells were cultured with splenocytes, the proliferation and expansion of Clone-4 cells were significantly decreased in the presence of splenocytes of aged mice compared to splenocytes of young mice (Fig. 5A: Young vs aged: 48h: 74.9 ± 6.9% vs 41.2 ± 5.7%, $p < 0.05$; 72h: 86.5 ± 7.6% vs 51.8 ± 8.2%, $p < 0.05$). Phenotypic analysis of the splenocytes showed that the percentage of DCs (CD11c$^+$MHC II$^+$ cells) in the spleen of aged mice was not significantly different than that of young mice (Fig. 5B: Young vs aged: 4.6% ± 0.35 vs 5.3% ± 0.6, $p > 0.05$), although the number of DCs in the entire spleen of aged mice was significantly lower than of young mice (Fig. 5C: Young vs aged: 2.7 ×10^6 ± 0.3 vs 1.7 ×10^6 ± 0.3, $p < 0.05$). In addition, the levels of expression of CD209, CD40, CD80, and CD86 on DCs were similar between young and aged mice (data not shown). Phenotypic analysis also indicated that the percentages of DC subpopulations: CD11b$^+$CD11c$^+$ (mDCs), CD8$^+$CD11c$^+$CD11b$^-$ (LyDCs), and B220$^+$CD11c^{low}CD11b$^-$ (pDCs) were comparable in young and aged mice (data not shown).

Splenic DCs were enriched by positive selection with CD11c$^+$, causing an increase of DCs (MHCII$^+$CD11c$^+$) to 47 ± 1.5 % of live cells in young and 50 ± 1.8% in aged mice. Individually, the frequencies of both MHC II$^+$ cells and CDllc$^+$ cells were higher in splenocytes from aged mice, with this disparity remaining after enrichment. The frequencies of CD8$^+$ cells and B220$^+$ cells were similar in splenocytes and the enriched DCs, while CD4$^+$ cells decreased by about half in both young and aged after enrichment. The loss of Treg cells (CD4$^+$CD25$^+$foxp3$^+$) after enrichment was more pronounced in preparations of aged than young mice: There was about a 50% loss of Treg cells in young from 7.2% to 3.4%, and a 90% loss in aged, from 11.1% to 1.0% of CD4$^+$ cells (Table 2).

To further examine whether the function of APCs in priming CD8 T cells is altered with aging, we incubated purified CFSE-labeled Clone-4 TCR-Tg T cells with HA peptide and this enriched DC preparation from young or aged wt BALB/c mice. The proliferation and expansion of the Clone-4 cells was comparable after culture with DCs of young and aged mice at both 48 h and 72 h post stimulation (Fig. 5D: Young vs aged: 48h: 83.1 ± 0.3% vs 82.2 ± 0.8%, $p > 0.05$; 72h: 95.8 ± 0.5% vs 95.7 ± 0.4%, $p > 0.05$).

To determine whether this phenomenon was mouse strain specific, we performed a similar series of experiments with B6 and P14 TCR Tg mice. Comparable to BALB/c mice, culture of purified P14 CD8 T cells with equal numbers of splenocytes of young or aged mice

demonstrated that splenocytes of aged B6 mice did not support proliferation of P14 CD8 T cells as effectively as splenocytes of young B6 mice (Fig. 5E: Young vs aged: 72h: 92.6% ± 1.5% vs 26.1% ± 1.1%, $p < 0.05$), but culture of P14 CD8 T cells with equal numbers of purified DCs of young or aged B6 mice demonstrated similar proliferation (Fig. 5H: 72h: Young vs aged: 82.9 ± 0.6% vs 80.3% ± 0.9%, $p > 0.05$). Similar to BALB/c mice, B6 mice demonstrated a significantly decreased number of DCs, with a comparable or even increased percentage of DCs in the spleen of aged compared to young mice (Fig. 5F & G). Taken together, these in vitro data demonstrate while the Ag presenting function of DCs of aged mice remains intact, there is a decrease in the total number of DCs in the spleen which in conjunction with other inhibitory population(s) may result in the poor priming of specific CD8 T cells of TCR Tg mice.

4. Discussion

To determine the effect of the aged environment on the Ag-specific CD8 T cell response to virus infection, the following questions were addressed in this study using two different genetic strains of mice (B6 and BALB/c mice) and two different viruses (influenza virus PR8 and LCMV Arm): 1) What is the difference in Ag-specific CD8 T cell responses to virus infection between young and aged mice? 2) Does the aged environment limit the clonal expansion of specific CD8 T cells after infection with virus? 3) Does the number and Ag presenting function of DCs change with aging?

Similar to our previous study of primary influenza infection in B6 mice (Po et al., 2002), a decreased Ag-specific CD8 T cell response occurred in both BALB/c and B6 aged mice after infection with influenza virus. While many studies suggest that this decreased response is due to intrinsic changes in T cells, the contribution of extrinsic factors has not been established. Plowden et al (Plowden et al., 2004) found that the Ag presenting function of macrophages of aged mice was defective in inducing Ag-driven naïve CD8 T cell clonal expansion, while Mittler et al (Mittler et al., 2004a and 2004b) reported that the aged lymphoid microenvironment inhibits memory, but not primary, CD4 T cell response. More recently, however, Tesar et al (Tesar et al., 2006) showed that adoptively transferred virus-specific young CD8 T cells responded equally regardless of the host's age, suggesting no age-associated alteration of the environment.

Due to the questions remaining regarding the ability of the aged environment to support T cell proliferation and function, we decided to explore this subject further. We chose to use CD8 T cells from TCR Tg mice in an adoptive transfer approach to explore whether the aged environment has an effect on Ag-specific CD8 T cell response to virus infection. Since TCR-Tg CD8 T cells exhibits high affinity to engage the epitope loaded in the MHC I molecule (Boulter et al., 2007; Pitcher et al., 2003), the expansion of the Tg T cells should be quicker and stronger than the virus-specific response of CD8 T cell of wt mice. Our results clearly demonstrate that some component(s) of the aged environment limit Ag-specific T cell response early after virus infection (Fig. 2 to 4). While our data show this negative impact of the aged environment in two virus models (influenza and LCMV), in two genetic backgrounds (BALB/c and B6) mice, and using two doses of adoptively transferred Tg T cells suggesting a consistent observation, our data with the P14 model was in direct conflict with the results of Tesar et al (Tesar et al., 2006) who reported no effect of the environment with a similar adoptive transfer model. Comparison of the two experiments revealed a major difference: although we both adoptively transferred large numbers of Tg CD8 T cells, we assessed response on Day 3 and they evaluated response on Day 8 after infection. We could not directly assess Day 7 in our P14 model, because we used the commercially available P14, Thy1.2 mice. Since the recipient B6 mice are also Thy1.2, we could not differentiate the response of donor vs recipient CD8 T cells on Day 7. Tesar et al were able to perform this assessment since they bred the P14 Tg

onto a Thy1.1 background. However, to address the hypothesis that the difference between the two studies was due to the kinetics of the response, we utilized the Clone-4 system in which the Thy1.1/Thy1.2 distinction between responding CD8 T cells could be made. When higher numbers of Clone-4 cells were transferred, we found minimal expansion of Tg CD8 T cells in either young or aged recipients on Day 2 after infection (< 0.1% of total CD8 T cells were IFN-γ^+). We consistently found a significantly decreased expansion of the transferred Tg CD8 T cells in aged mice on Day 3 (Young vs aged: 23% vs 11%; Fig. 2). By Day 7, the percent of CD8 T cells in young mice that were $HA^+Thy1.1^+$ decreased to 9.7%, while aged mice demonstrate a smaller decrease to 9.3% $HA^+Thy1.1^+$. These results reflect the kinetics we previously reported after infection of wt mice in which young mice demonstrate an earlier peak of response. When the response is evaluated at the peak of response of the aged, the level of response appears comparable between young and aged mice, since the response of young had already contracted (Po et al., 2002). Although not a direct assessment of the difference between the results of Tesar et al and ourselves, these data in the Clone-4 system suggest that the lack of an age-associated difference observed by Tesar et al (Tesar et al., 2006) could be due to the fact that the peak response in young had already contracted by Day 8, while the response of the aged mice had just reached its maximum. Our data in the Clone-4 system, using lower numbers of transferred Tg T cells, further support the interpretation that the difference in the two studies is due to kinetics. On Day 4 the response in young is 19×10^4 and increases tenfold to 18×10^5 on Day 7. In aged mice the response also increases tenfold from 4.8×10^4 to 6.8×10^5 on Day 4 to Day 7. The expansion of the lower numbers of specific Tg CD8 T cells was significantly decreased in aged compared to young mice on both Day 4 and Day 7, suggesting that with the lower numbers of Tg cells adoptively transferred the peak response in aged mice had not been reached by Day 7.

An interesting observation of the adoptive transfer model is seen in Fig. 4A. Without infection, young and aged recipients demonstrate a comparable percentage of CFSE-labeled P14 cells. Upon infection, there is a proliferation of these virus-specific TCR Tg CD8 T cells resulting in a decreased percent of CFSE labeled cells. In young hosts, this proliferation results in an increase in virus-specific (GP^+), but CFSE negative, CD8 T cells. However, in aged recipients, the percent of CFSE labeled CD8 T cells decreased with only a limited increase in virus-specific CFSE negative CD8 T cells. This loss of CD8 T cells that are responding to the virus suggests apoptosis maybe occurring due to the aged environment. The mechanism of this age-associated loss of virus-specific CD8 T cells requires further examination.

DCs play a pivotal role in generating both innate and adaptive immunity to infection (Lanzavecchia et al., 2001; Sallusto et al., 2002; Iwasaki et al., 2007). However, studies regarding DC functions and aging are limited, and the results are conflicting. No consensus has been reached concerning the alteration of the number nor the function of DCs in aged mice or individuals (Tesar et al., 2006; Linton et al., 2001; Shurin et al., 2007). When we used equal number of splenocytes as APCs, the priming of specific CD8 T cells in two virus systems is decreased in the presence of splenocytes from aged mice. This limited expansion in the presence of splenocytes of aged mice may be due to 1) decreased number of APCs; 2) decreased function of APCs; or 3) both. When equal numbers of purified DCs from young or aged mice were cultured with virus-specific CD8 T cells of young mice, the clonal proliferation and expansion of CD8 T cells were similar, indicating that the priming function of DCs from aged mice remains intact in vitro regardless of BALB/c or B6 background. This is supported by a previous study that showed that the functions of DCs from aged mice remain intact even though there exists slight differences in the percentages of myeloid vs lymphoid DCs and surface expression of MHC molecules in DCs between young and aged mice (Norian et al., 2004). This suggested that the decreased expansion with comparable number of splenocytes was due to the number of DCs of aged mice. However, phenotypic analysis indicated that the percentage of DCs as defined by MHC II^+CD11c^+ or by $CD11b^+CD11c^+$ (mDCs),

$CD8^+CD11c^+CD11b^-$ (LyDCs), and $B220^+CD11c^{low}CD11b^-$ (pDCs) is comparable if not slightly higher in aged mice. The limited expansion of Tg CD8 T cells in the presence of comparable splenocytes suggests the presence of another cellular factor in aged mice that inhibits the proliferative response, possibly the increased percentage of CD4 Treg cells (Sharma et al., 2006).

In addition to DCs, B cells and macrophages also have the ability to present Ag to T cells. It was reported that macrophages of aged mice exhibit defects in APC function resulting in impaired Ag-induced CD8 T cell clonal expansion in vitro (Plowden et al., 2004). Few studies have been reported on the alteration of Ag presenting function of B cells with aging during virus infection. In a preliminary study we performed, purified B cells of aged mice were equally capable as B cells of young mice of supporting specific CD8 T cell expansion in vitro (data not shown).

The decrease in immunological function with aging is an important process in which multiple extrinsic and intrinsic factors may be involved. Although age-related alterations of T cell function have been extensively studied, the studies on the potential changes of host environment with aging are very limited. Our findings suggest that factors of the aged-microenvironment play an important role in the alteration of Ag-specific CD8 T cell response to virus infection. Further examination of the aged environment is needed in order to better understand the mechanisms of immune senescence with aging, and to improve the efficacy of immunization against viral infections in aged individuals.

Abbreviations used

HAU	hemagglutination unit
LCMV	lymphocytic choriomeningitis virus
DC	dendritic cell
Tg	transgenic
NP	$NP_{366–374}/D^b$
HA	$HA_{518–526}/K^d$
GP	$GP_{33–41}/D^b$

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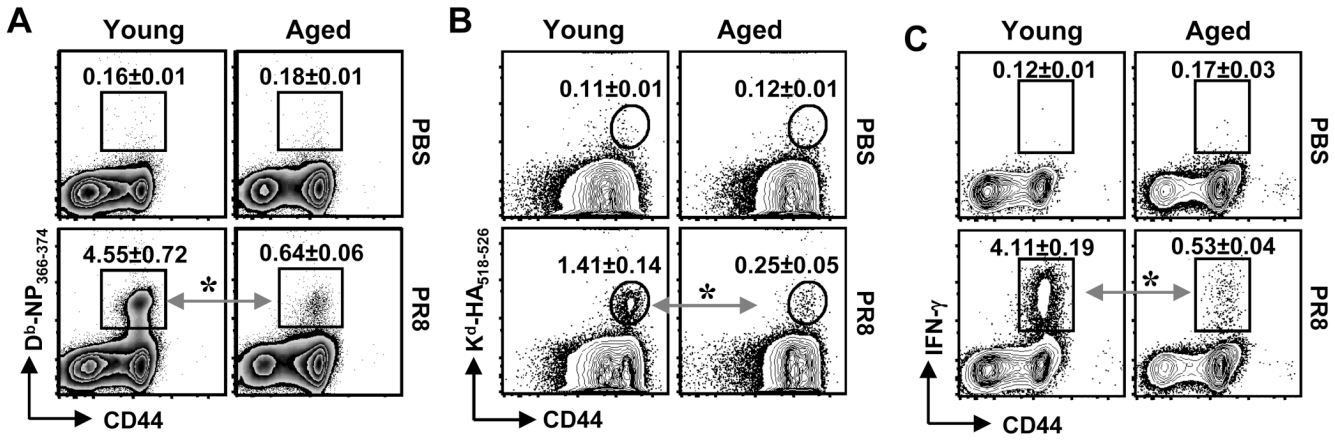

Fig. 1.

Ag-specific CD8 T cell response to primary influenza infection is decreased in aged mice. Young and aged B6 (A & C), and BALB/c (B) mice were infected i.v. with 300 HAU PR8. On Day 7 post infection, splenocytes were isolated, stained with CD8, CD44 and anti-IFN-γ antibodies, as well as D^b-$NP_{366–374}$, or K^d-HA tetramers, and assessed by flow cytometry. Fig. A and B: $Tetramer^+/CD44^{high}$ cells are presented as percent of total CD8 T cells. Fig. C: IFN-γ production as percentage of cells that are $CD8^+IFN\text{-}\gamma^+$ of total $CD8^+$ cells. Each plot shows $CD8^+$ cells from one representative mouse with numbers indicating mean ± SD of 3–4 mice. * $p < 0.05$. Data are representative of three independent experiments with similar results.

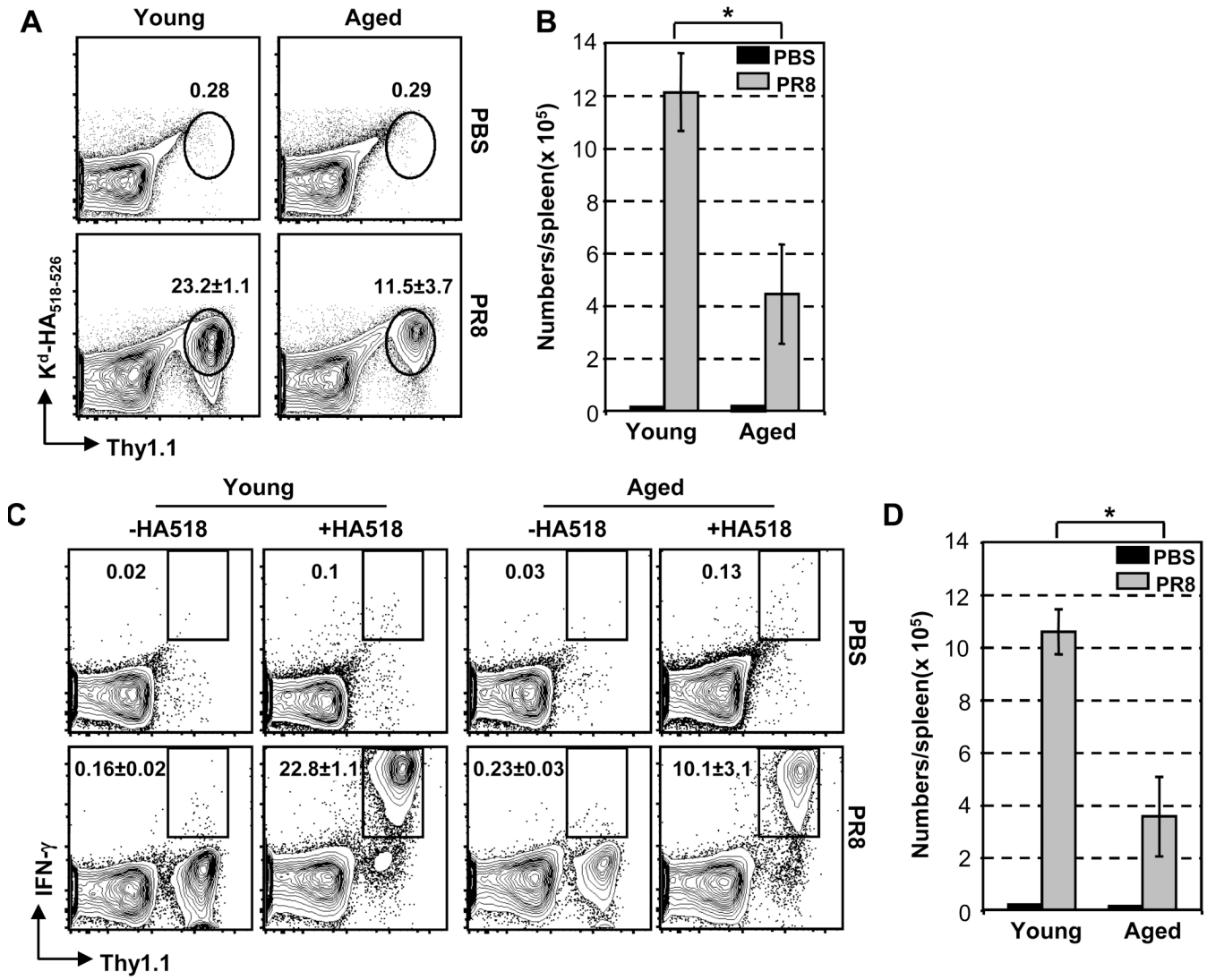

Fig. 2.

Limited expansion of higher numbers of Tg CD8 T cells in response to influenza virus infection in aged BALB/c mice. 2–4 × 10^6 splenocytes of Clone-4 TCR-Tg mice (Thy1.1$^+$) were adoptively transferred into congenic young and aged BALB/c mice (Thy1.2$^+$). Recipient mice were infected i.v. with 300 HAU PR8. On Day 3 post-infection, splenocytes were isolated, stained with antibodies to CD8, CD44 and IFN-γ, as well as K^d-HA tetramer. Donor CD8 T cells were identified by HA$^+$/Thy1.1$^+$ staining after gating on total CD8 T cells. (A) Percentages of HA-specific donor CD8 T cell of total CD8$^+$ cells. (B) Absolute numbers of $HA_{518–526}$$^+$/Thy1.1$^+$ cells. (C) Percentage of Thy1.1$^+$IFN-γ$^+$ gated on CD8$^+$ cells. (D) Absolute numbers of Thy1.1$^+$IFN-γ$^+$ cells. Each plot is representative of one mouse with numbers reflecting ± SD of 3–4 mice. * $p < 0.05$. These results are representative of four independent experiments with similar results.

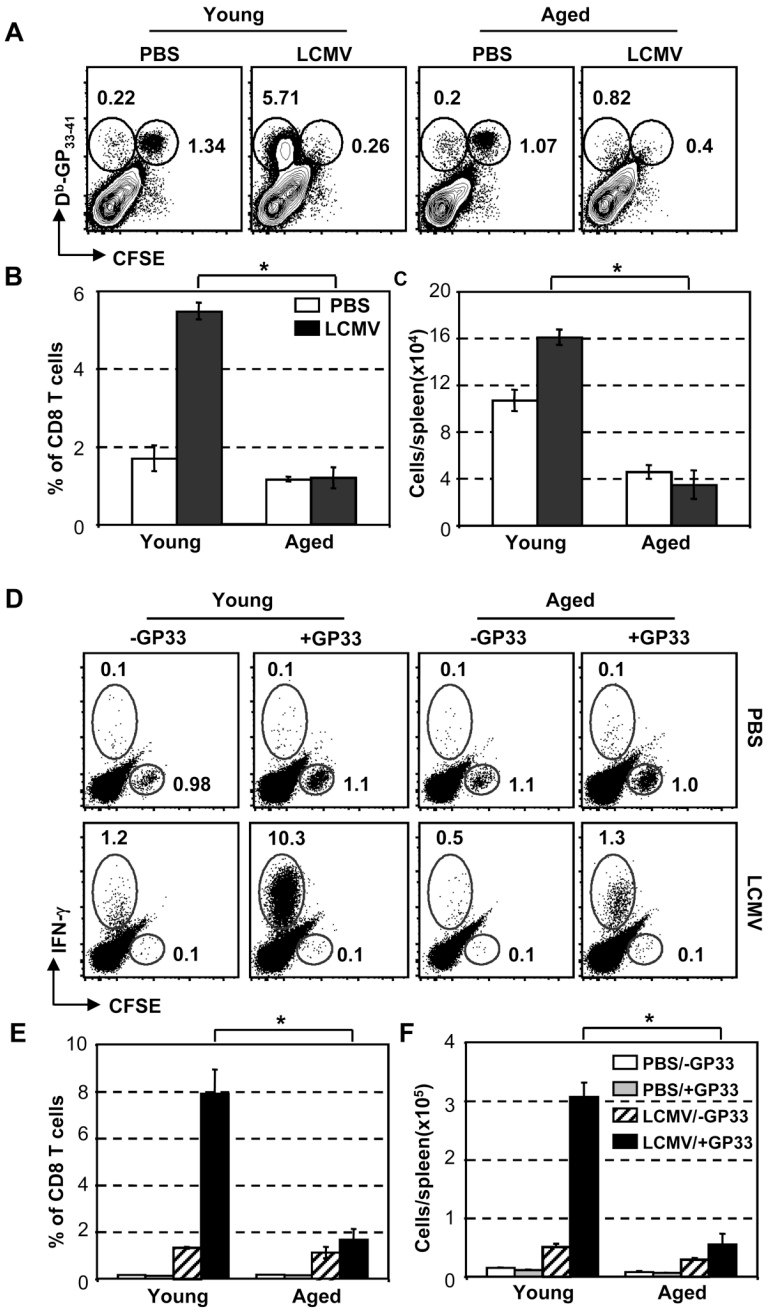

Fig. 3.

Limited expansion of D^b-$GP_{33–41}$ Tg CD8 T cells in aged B6 mice after infection with LCMV. 2–4 × 10^6 CFSE-labeled splenocytes from $GP_{33–41}$-specific P14 T B6 mice were adoptively transferred into young and aged B6 mice. Recipient mice were infected i.p. with 2 ×10^6 PFU LCMV Arm. On Day 3 post-infection, proliferation and expansion of transferred P14 cells in the spleen were examined by CFSE, antibody and D^b-$GP_{33–41}$ tetramer staining. (Fig. A & B) Percentages of $GP_{33–41}^+$ specific CD8 T cells of total $CD8^+$ cells; (C) Absolute numbers of $GP_{33–41}^+$ CD8 T cells (gated on $CD8^+$ cells); (Fig. D & E) Percentages of IFN-γ^+ CD8 T cells, gated on $CD8^+$ cells; (F) Absolute numbers of $CD8^+$IFN-γ^+ cells. Each plot is representative

of one mouse from 3–4 mice per group. Error bars represent ± SD. * $p < 0.05$. Similar results were obtained in three separate experiments.

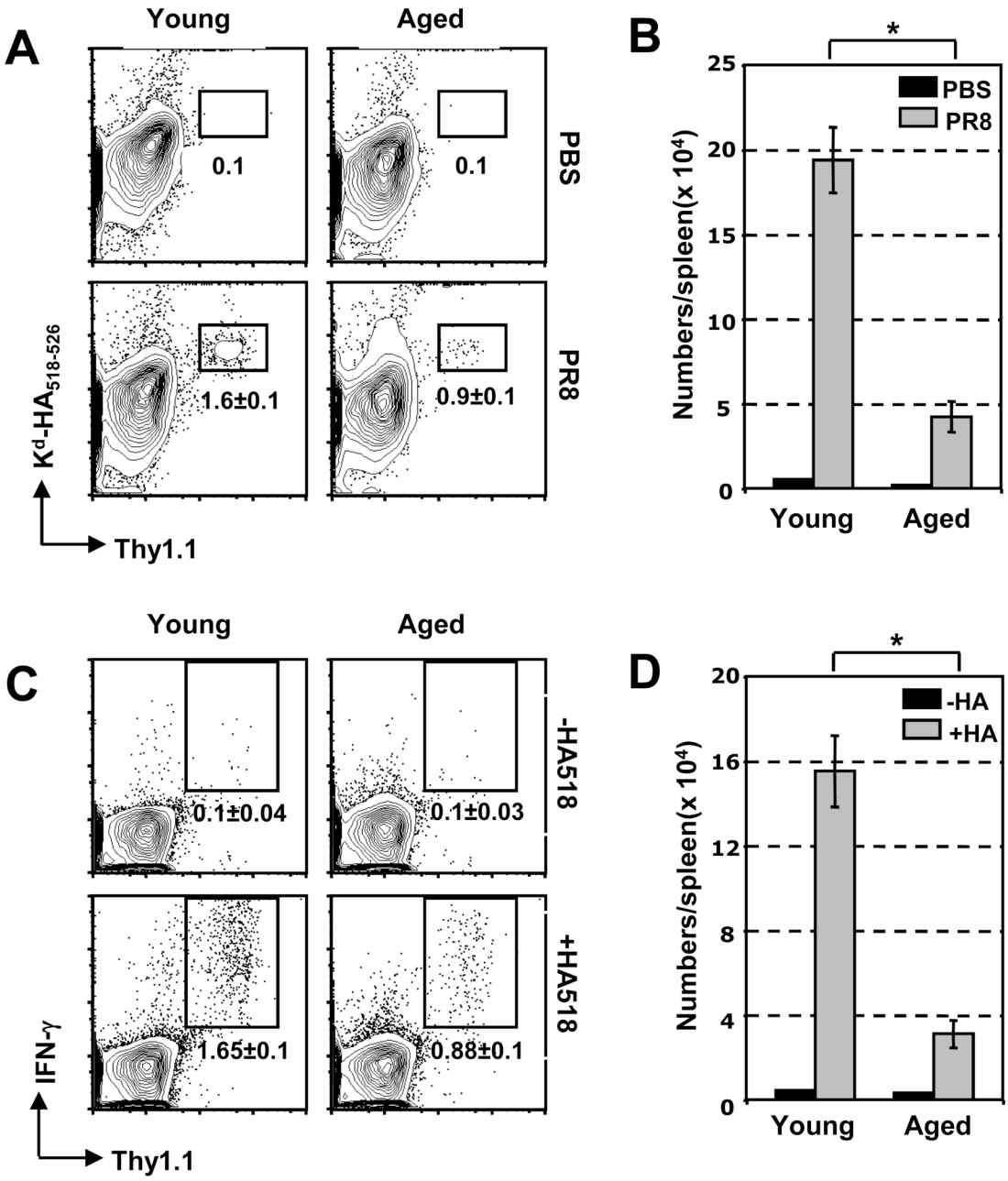

Fig. 4.

Limited expansion of lower numbers of $HA_{518–526}$ Tg CD8 T cells in response to influenza virus infection in aged BALB/c mice. 5×10^3 splenocytes of Clone-4 cells ($Thy1.1^+$) were adoptively transferred into young and aged BALB/c mice ($Thy1.2^+$). The recipients were infected i.v. with 300 HAU PR8. On Day 4 after infection, expansion of donor CD8 T cells in the spleens was identified by $HA^+Thy1.1^+$ staining after gating on total CD8 T cells, and the function of the specific CD8 T cells were examined by intracellular IFN-γ staining after in vitro stimulation with $HA_{518–526}$ peptide for 5 h. (A) Percentages of HA-specific donor CD8 T cell of total $CD8^+$ cells. (B) Absolute numbers of $HA_{518–526}{}^+Thy1.1^+$ cells. (C) Percentage of $Thy1.1^+IFN\text{-}\gamma^+$ gated on $CD8^+$ cells. (D) Absolute numbers of $Thy1.1^+IFN\text{-}\gamma^+$ cells. Each

plot is representative of one mouse with numbers reflecting ± SD of 3–4 mice. * $p < 0.05$. Similar results were obtained in three independent experiments.

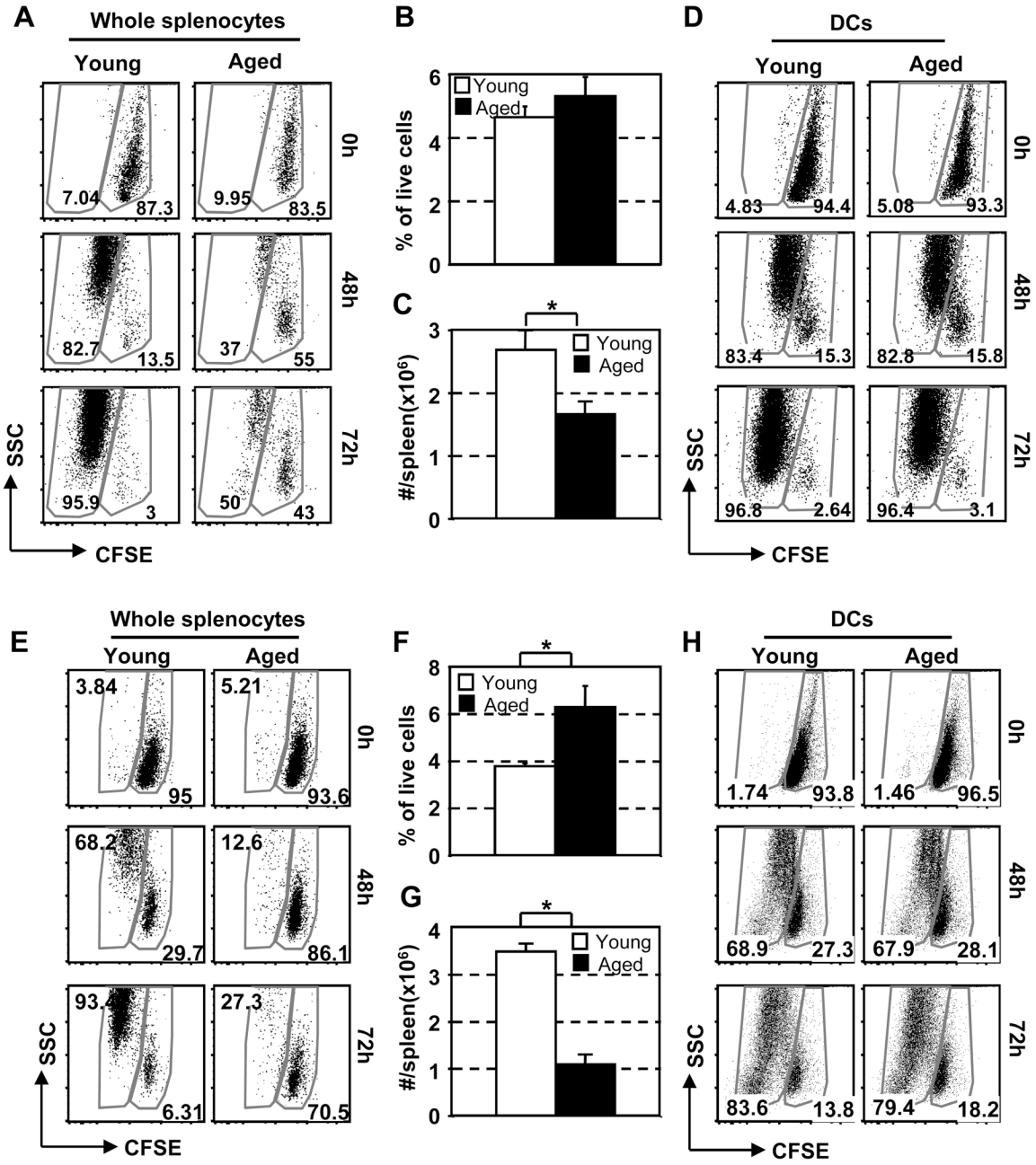

Fig. 5.

The ability of splenocytes of aged mice to support expansion of specific CD8 T cells is lower compared to splenocytes of young mice. (A–D) Clone-4 Tg mice and BALB/c mice: (A) 1×10^5 CFSE-labeled purified Clone-4 CD8 T cells were cultured with $HA_{518–526}$ and 5×10^5 splenocytes from young or aged BALB/c mice. Proliferation and expansion of the TCR-Tg T cells (gated on $Thy1.1^+CD8^+$); (B) Percentages, and (C) Numbers of $CD11C^+$/MHC $I\text{-}A^{d+}$ cells in the spleen of BALB/c mice; (D) 1×10^5 CFSE-labeled Clone-4 CD8 T cells ($Thy1.1^+$) were cultured with 1×10^4 enriched DCs from young or aged BALB/c mice ($Thy1.2^+$). Proliferation and expansion of the TCR-Tg T cells. (E–H): P14 Tg mice and B6 mice: (E) 1×10^5 CFSE-labeled purified P14 CD8 T cells were cultured with 5×10^5

splenocytes from young or aged B6 mice. Proliferation and expansion of the P14 cells (gated on $CD8^{+}GP_{33–41}{}^{+}$); (F) Percentages, and (G) Numbers of $CD11C^{+}$/MHC $I\text{-}A^{b+}$ cells in the spleen of B6 mice; (H) 1×10^{5} CFSE labeled P14 CD8 T cells were incubated with 1×10^{4} enriched DCs from young or aged B6 mice. Proliferation and expansion of P14 cells. N = 3 in each group. Error bars represent ± SD. * $p < 0.05$. Data are representative of three independent experiments with similar results.

Table 1

IFN-γ Production by $NP_{366–374}^{+}$ CD8 T Cells in Spleen[a]

		$NP_{366–374}^{+}$		IFN-γ^{+}	
		Total	**IFN-γ^{+}**	**Total**	**$NP_{366–374}^{+}$**
Young	PBS	0.7 ± 0.1	--	0.07 ± 0.01	--

		$NP_{366–374}^+$ IFN-γ^+		IFN-γ^+ $NP_{366–374}^+$	
		Total		Total	
Aged	D7	5.5 ± 0.3	57.3 ± 0.3	3.3 ± 0.2	89.9 ± 0.6
	D10	2.6 ± 0.1	43.0 ± 2.3	1.4 ± 0.1	75.5 ± 4.5
	D14	2.5 ± 0.4	46.4 ± 2.5	1.6 ± 0.3	72.0 ± 1.2
	PBS	0.7 ± 0.1	--	0.3 ± 0.1	--
	D7	0.7 ± 0.2	36.1 ± 4.0	0.5 ± 0.04	48.1 ± 11.7

	$NP_{366–374}^{+}$		IFN-γ^{+}	
	Total	**IFN-γ^{+}**	**Total**	**$NP_{366–374}^{+}$**
D10	1.9 ± 0.3	46.5 ± 5.3	1.9 ± 0.5	64.6 ± 6.3
D14	1.3 ± 0.2	34.2 ± 6.5	0.9 ± 0.3	67.1 ± 5.4

[a] N = 3–4 B6 mice in each group at each time point. Error bars represent ± SD.

[*] $p < 0.05$. Data are representative of two independent experiments with similar results.

Table 2

Phenotyping of splenocytes from B6 mice before and after DC enrichment

	Young		Aged	
	before	**after**	**before**	**after**
$CD11c^+$	9.3	66.6	15.2	78.5
DC ($CD11c^+/MHCII^+$)	4.6	48.7	7.9	50.6
$CD25^+/foxp3^{+*}$	7.2	3.4	11.1	1.0
$CD8^+$	14.6	16.4	9.8	12.1
$CD4^+$	24.8	9.7	18.0	6.9
$B220^+$	52.6	47.6	49.6	43.8

*Numbers represent percent of live cells, except, which represents percent of $CD4^+$ cells.