CD4^{+}CD25^{+}Foxp3^{+} T Cells and CD4^{+}CD25^{-} Foxp3^{+} T Cells in Aged Mice

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CD4⁺CD25⁺Foxp3⁺ T Cells and CD4⁺CD25⁻Foxp3⁺ T Cells in Aged Mice

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Aging is associated with a progressive decline in T cell-mediated immune responses. However, it has been unknown whether regulatory/suppressive CD4 T cells are involved in this decline. Our in vitro analyses revealed that CD4⁺CD25⁺ T cells, the well-characterized naturally occurring regulatory/suppressive CD4 T cells, in aged mice are functionally comparable to those in young mice (i.e., anergic and suppressive), although slightly increased in number. In contrast, functional changes to whole CD4⁺CD25⁻ T cells were pronounced in aged mice, i.e., the majority of aged CD4⁺CD25⁻ T cells exhibited a significant hypo-responsiveness, and the remaining cells maintained a normal responsiveness. Furthermore, we identified Foxp3 (a transcription factor critical in conferring the regulatory/suppressive function to CD4 T cells)-positive suppressive CD4 T cells among aged hyporesponsive CD4⁺CD25⁻ T cells. These results suggest that the age-related decline in T cell-mediated immune responses is ascribable to changes in the CD4⁺CD25⁻ T cell population and not to a functional augmentation of suppressive CD4⁺CD25⁺ T cells.

FIGURE 1. CD4+CD25high T cells in aged B6 mice maintain their suppressive function. A, Spleen cells from various aged B6 mice (2, 12, and 24 mo old) were stained with FITC-anti-CD4 Ab, biotinylated anti-CD25 Ab, and PE-conjugated streptavidin. A representative staining of B6 (2 mo old) spleen cells with anti-CD4 Ab (ordinate) and anti-CD25 Ab (abscissa) (logarithmic scale). The gates for whole CD25+ cells (R2) and CD25 highly positive cells (R3; designated as CD25high) are indicated. B, Spleen cells were stained with FITC-anti-CD25 Ab and PE-anti-CD4 Ab. Each symbol represents the percentage of CD4+CD25+ cells (corresponding to R2) among all CD4+ cells for individual mice. C, Spleen cells prepared from young (2 mo: bold line) or aged (24 mo: thin line) B6 mice were stained with FITC-anti-CD4 Ab, biotinylated anti-CD25 Ab plus PE-conjugated streptavidin, and allophycocyanin-anti-Foxp3 Ab. All CD4+CD25+ cells (that is cells in R2 in Fig. 1A, left panel) and CD4+CD25high cells (R3, right panel) were analyzed for Foxp3 expression. Cells were stained with anti-CD4, anti-CD25, and isotype-matched control Ab as a control (dotted line). D, CD4+CD25+ T cells (1 × 10^6/well, purified from 2-mo-old B6 mice) mixed with either of the titrated number of CD4+CD25high T cells (R3 in Fig. 1A) from 2- or 24-mo-old B6 spleen cells (△ and □, respectively) were cultured for 3 days with anti-CD3 Ab (final 5% of SN) in the presence of young B6 splenic APC (20 Gy irradiated). Cell proliferation was measured on culture day 3. Young CD4+CD25+ T cells, young CD4+CD25high T cells, and aged CD4+CD25high T cells cultured as well gave 78,242, 126, and 633 cpm, respectively, in a representative experiment. A result representative of three independent experiments is shown.

In vitro proliferation assay

The sorted T cells (1 × 10^6/well) were stimulated for 3 days with anti-CD3 (145-2C11) Ab SN, along with irradiated (20 Gy) young B6 spleen cells as APC (5 × 10^6/well), in 96-well round-bottom plates in DMEM supplemented with 10% FCS. The incorporation of [3H]thymidine (37 kBq/well) (DuPont/NEN) by proliferating T cells (triplicate cultures) during the last 6 h of the culture was measured. Data are the mean ± SD for triplicate wells.

Cell staining

The intracellular staining of mouse splenocytes with anti-Foxp3 Ab was done with the use of an anti-mouse Foxp3 staining set (eBioscience) according to the manufacturer’s directions. For rhodamine-123 (R123; Molecular Probes) staining, cells were incubated with 12.5 mM R123 for 10 min at 37°C, washed three times with cold PBS, incubated for an additional 30 min at 37°C, and washed. In some experiments, the stained cells were further stained with PE-anti-CD103 Ab (BD Pharmingen).

RNA analysis

For the quantitative analysis of mRNA expression, RNA was extracted from the samples using Isogen reagent (Nippon Gene), and cDNA was synthesized using standard procedures. The expression of Foxp3 and hypoxanthine phosphoribosyltransferase (HPRT) was determined by quantitative real-time PCR using the primers below. Expression was normalized to the level of HPRT in each sample: Foxp3, 5'-CCCAGGAAGCAGCACCTT-3' and 5'-TTTCTCAACCGGCCACTTG-3'; and HPRT, 5'-TGAGAGCTACTGTAATGATCAGTCAAC-3' and 5'-AGCAAGCTTGCAACCCTTAACCA-3'.

Cytotoxicity assay

Spleen cells (5 × 10^6 cells in 2 ml) were cultured for given periods in 24-well plates (Costar). Viable cells were harvested and used as effectors in the 51Cr release cytotoxicity assay, in which target cells (1 × 10^4) were labeled with 5.7 MBq of sodium chromate (DuPont/NEN) for 60 min at 37°C, washed three times, and incubated at 1 × 10^4 cells/well with indicated numbers of effector cells in 96-well round-bottom plates (Costar) for 6 h at 37°C. The mean percentage specific lysis of duplicate cultures was calculated from the radioactivity of the SN: % specific lysis = 100 × (cpm experimental release – cpm spontaneous release)/cpm maximal release – cpm spontaneous release). Spontaneous release from the target cells incubated in medium alone was always <15% of the maximal release obtained by adding 1 N HCl to the labeled target cells.

Tumor cells

B16 (B6-derived melanoma) (23) and RLMale1 (BALB/c-derived radiation leukemia) (24) used as target cells in cytotoxicity assays were gifts from

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*AAbbreviations used in this paper: SN, supernatant; R123, rhodamine-123; HPRT, hypoxanthine phosphoribosyltransferase.
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Results

**CD4^+CD25^{high}Foxp3^{+} T cells in aged mice maintain their suppressive function**

It is well recognized that aging leads to a decline in the ability to mount normal CD4 T cell responses (20–22). This observation raises the possibility that the functional deterioration of whole CD4 T cells may result from a functional imbalance (qualitative or quantitative) of CD4^+CD25^{+} T cells in aged hosts, which were recently reported as a T cell-subpopulation functioning to down-modulate immune responses (1–3). To investigate this possibility, we first compared the number of CD4^+CD25^{+} T cells from B6 mice of different ages. The staining of B6 (2 mo old) spleen cells with anti-CD4 and anti-CD25 Ab is shown in Fig. 1A. In aged mice, we observed slightly increased total numbers of spleen cells (2 mo old: 901 × 10^5 ± 371 × 10^5, n = 5; 12 mo old: 982 × 10^5 ± 194 × 10^5, n = 7; 24 mo old: 1083 × 10^5 ± 287 × 10^5, n = 6). No significant differences were observed in the proportion of total CD4^+ T cells from the spleens of 2-mo-old (24.2 ± 4.8%, n = 7), 12-mo-old (20.5 ± 5.0%, n = 10), or 24-mo-old (22.3 ± 3.8%, n = 9) B6 mice. In contrast, as shown in Fig. 1B, the proportion of CD25-expressing cells (R2 in Fig. 1A) among CD4^+ T cells from 24-mo-old mice was slightly larger (~1.7-fold), as compared with that among CD4^+ T cells from 2-mo-old mice (2 mo old: 9.1 ± 1.0%, n = 7; 12 mo old: 11.7 ± 1.9%, n = 10; 24 mo old: 15.6 ± 2.5%, n = 9).

The expression of CD25 on CD4 T cells is induced upon T cell activation. Therefore, one possibility is that the CD4^+CD25^{+} cell population contains both suppressive CD4^+CD25^{+} T cells and nonsuppressive/temporary-activated CD4 T cells derived from CD4^+CD25^{−} T cells. To exclude this possibility, we analyzed the expression of Foxp3 inside cells. Foxp3 is a transcription factor...
conferring a suppressive function to CD4 T cells (17–19). As shown in Fig. 1C, left panel, almost all CD4+CD25+ T cells prepared from young mice expressed Foxp3 (Foxp3-positive cells in the CD4+CD25+ cell fraction from young B6 mice: 81.9 ± 2.1%), n = 4). In contrast, aged CD4+CD25+ T cells included Foxp3+ and Foxp3– cells (Foxp3-positive cells in the CD4+CD25+ cell fraction from aged B6 mice: 65.4 ± 9.5%, n = 4), indicating that the aged CD4+CD25+ T cell population contains activated T cells, i.e., temporary CD25+ T cells, derived from CD4+CD25– T cells. However, the level of Foxp3 expression on CD4+CD25+ highly positive cells (designated CD4+CD25high T cells, corresponding to R3 in Fig. 1A) was similar between young and aged mice (Fig. 1B). Foxp3-positive cells among young CD4+CD25high T cells: 94.4 ± 1.4%, n = 4; aged CD4+CD25high T cells: 86.7 ± 2.3%, n = 4). The proportion of CD4+CD25high T cells among all CD4 T cells in young and aged mice was 7.7 ± 1.8% (n = 4) and 10.5 ± 3.5% (n = 4), respectively.

Next, we examined whether the CD4+CD25highFoxp3+ T cells from aged mice were functionally suppressive or not. The sorted CD4+CD25high T cells from young or aged mice were mixed with young CD4+CD25– T cells as responder cells at various ratios and cultured along with APC and anti-CD3 Ab. As shown in Fig. 1D, the same level of suppression by CD4+CD25high T cells from young or aged mice. Both CD4+CD25high T cells exhibited an anergic state following polyclonal stimulation. Taken together, these results demonstrate that CD4+CD25highFoxp3+ T cells retain their suppressive function even in aged hosts.

Functional comparison of CD4+CD25– T cells prepared from young or aged B6 mice

In the absence of suppressive CD4+CD25+ T cells, the remaining CD4+CD25– T cells exhibit a more efficient activation in response to polyclonal stimulation (4, 6). However, we found that this was not the case for aged CD4+CD25– T cells. CD4+CD25– T cells from young and aged mice were stimulated in various culture conditions (Fig. 2A). Young CD4+CD25– T cells were stimulated more efficiently under better culture conditions (higher concentration of anti-CD3 Ab and/or optimal number of APC per well). However, aged CD4+CD25– T cells exhibited poor responsiveness even in the best culture conditions for young CD4+CD25– T cells, contrasting to the maintenance of function in aged CD4+CD25high T cells (Fig. 1D).

CD4+CD25– T cells from aged mice exhibited hypo responsiveness in the proliferation assay (Fig. 2A). We next examined in vitro whether this is also the case in other functional assays. We have previously shown that the culture of CD4+CD25+ T cell-depleted splenic cell suspensions prepared from tumor-un sensitized normal young mice led to the spontaneous generation of cytotoxic cells capable of killing a broad spectrum of tumors (8). The generation of cytotoxic cells was ascribable to the production of IL-2 by CD4+CD25– T cells. This was reconfirmed in Fig. 2, B and C, demonstrating the spontaneous generation of cytotoxic cells when CD25– T cell-depleted spleen cells from 2-mo-old mice were cultured for 1 wk without tumor stimulation. In contrast, cytotoxic activity was not generated from cultures of CD25+ T cell-depleted spleen cells prepared from aged (24 mo old) spleen cells; similar cultures of CD25+ spleen cells from 12-mo-old mice generated intermediate levels of cytotoxic activity, depending on individual mice (Fig. 2, B and C). Furthermore, in accordance with our previous report (8), CD25+ T cell-depleted spleen cells from young (2 mo old) mice showed spontaneous proliferation and production of IL-2, which was mainly secreted by self-reactive CD4+CD25– T cells contained in the CD25– cell population, whereas CD25+ T cell-depleted spleen cells from 12- or 24-mo-old mice did not (data not shown). These results indicate that functional deteriorations occurred in CD4+CD25– T cells from aged mice.

Appearance of CD4+CD25–Foxp3+ T cells in aged mice

We next examined whether all aged CD4+CD25– T cells suffer some loss of function. To this end, we first investigated the expression of Foxp3 in all spleen cells. Foxp3-expressing cells belonged to the CD4 T cell population in young and aged mice (Fig. 3A). The proportion of Foxp3 cells among aged mice was increased significantly (Foxp3+ cells among young CD4 T cells: 14.1 ± 2.8%, n = 4; among aged CD4 T cells: 32.1 ± 5.1%, n = 4; p = 0.00079). Next, the expression of CD25 in Foxp3+ and Foxp3– CD4 T cells (R4 and R5 in Fig. 3A, respectively) was analyzed (Fig. 3B). Again, we confirmed that almost all young CD4+Foxp3+ T cells expressed CD25 (CD25+ cells: 73.7 ± 4.0%, n = 4) and that aged CD4+Foxp3+ T cells included CD25– and CD25+ cells, suggesting that the aged CD4 T cell population contains temporarily activated CD4+CD25highFoxp3+ T cells. In contrast with young CD4+Foxp3+ T cells, surprisingly, a significant proportion of CD4+Foxp3+ T cells from aged mice were CD25 negative (CD25-positive cells: 70.5 ± 6.1%, n = 4), suggesting the appearance and/or an increase in the number of CD4+CD25– Foxp3+ T cells with aging.

Aged CD4+CD25– T cells consist of functionally heterogeneous subpopulations

We have previously shown that aged CD4+CD25– T cells could be divided into three populations based on staining with R123 and anti-CD103 Ab (25). Both CD4+CD25– T cells from young and aged mice can incorporate R123 during a short period of exposure. However, following incubation for 30 min at 37°C under R123-free conditions, an obvious difference was observed between young and aged CD4+CD25– T cells, i.e., the majority of the aged

![FIGURE 3. Appearance of CD4+CD25 Foxp3+ T cells in aged B6 mice. A. Spleen cells prepared from young (2 mo) and aged (24 mo) B6 mice were stained with anti-CD4 and anti-Foxp3 Ab. B. Cells in R4 (bold line) and R5 (thin line) in A were analyzed for the expression of CD25. A result representative of four independent experiments is shown.](http://www.jimmunol.org/ by guest on October 27, 2017)
CD4+ Foxp3+ T cells in aged mice

We further examined the expression of CD25 on thymocytes prepared from aged mice (Fig. 5A). Although the thymus was very small in aged mice compared with young mice, the profile of CD25 expression in aged mice was almost the same as that in young mice (the staining profile of young thymocytes is shown in Ref. 26), i.e., CD4+ CD8- cell populations contained CD25+ cells; the intensity of CD25 expression was greater on cells in the CD4+ CD8- fraction than the CD4 single-positive fraction.

We next examined the Foxp3 expression in thymocytes from aged mice (Fig. 5B). Foxp3+ cells existed in only the CD4+ CD8- T cells (R6 in Fig. 4D).

Expression of Foxp3 in thymocytes from aged mice

We further examined the expression of CD25 on thymocytes prepared from aged mice (Fig. 5A). Although the thymus was very small in aged mice compared with young mice, the profile of CD25 expression in aged mice was almost the same as that in young mice (the staining profile of young thymocytes is shown in Ref. 26), i.e., CD4+ CD8- and CD4+ CD8- cell populations contained CD25+ cells; the intensity of CD25 expression was greater on cells in the CD4+ CD8- fraction than the CD4 single-positive fraction.

We next examined the Foxp3 expression in thymocytes from aged mice (Fig. 5B). Foxp3+ cells existed in only the CD4+ CD8- T cells. CD4+ CD25+ R123lowCD103+ T cells (R6 in Fig. 4A), CD4+ CD25+ R123lowCD103- T cells (R7), and CD4+ CD25+ R123high cells (R8) from aged mice were sorted, then the expression of Foxp3 protein in these three subpopulations was analyzed (bold lines). Control staining is shown as thin lines. E, Cells corresponding to R6, R7, and R8 in young CD4+ CD25+ cells (A, left panel) were sorted, and the expression of Foxp3 protein was analyzed as in D. A result representative of three to five independent experiments is shown.
fraction. To investigate the relation between Foxp3 and CD25 expression in thymocytes from aged mice, CD4+CD8+ thymocytes were stained with anti-CD25 and anti-Foxp3 Ab. CD4+CD8+CD25high thymocytes also expressed Foxp3 (73.8% Foxp3 positive in a representative experiment), but CD4+CD8+CD25low thymocytes did not (Fig. 5C), similar to young thymocytes (27). Taken together, these results show that the relation between CD25 and Foxp3 as a marker is maintained in the thymus even in aged mice, whereas the use of CD25 as a marker of suppressive CD4 T cells in the periphery is reduced in aged mice.

Discussion

It is well established that CD4 T cell function in response to Ag declines with aging (20). In this report, by dividing aged CD4 T cells into subpopulations, we showed that a suppressive CD4 T cell population (CD4+CD25high T cells) and part (CD4+CD25– R123high T cells) of a nonsuppressive CD4 T cell population (CD4+CD25– T cells) maintain their suppressive and normal responsive function, respectively, and a majority (CD4+CD25– R123low T cells) of CD4+CD25+ T cells exhibit hyporesponsiveness (R6 in Fig. 4B and R7 in Fig. 4C), and these hyporesponsive CD4 T cells (CD4+CD25– R123low T cells) include a suppressive CD4 T cell subpopulation (CD4+CD25– R123highCD103+Foxp3+ T cells) that was negligible in the young. Approximately 10% of CD4+CD25– R123highCD103+ T cells were Foxp3+ (R7 in Fig. 4D), and young whole CD4 T cells also contain ~10% Foxp3+ cells (Fig. 3A). However, the former exhibited a significant low responsiveness (Fig. 4C) compared with the normal responsiveness by the latter. Based on these results, it is likely that a remarkable low responsiveness of aged CD4+CD25– R123highCD103+ T cells results from hyporesponsiveness of Foxp3+ cells in aged CD4+CD25– R123lowCD103+ T cells and not from a suppressive function of Foxp3+ cells included in aged CD4+CD25– R123lowCD103+ T cells.

Regarding the relationship between the thymus and aging, dramatic changes in the thymic microenvironment with aging, referred to as thymic involution, have long been considered the most reasonable cause of the age-related decline in T cell function (20–22). However, of particular importance in this study is that, even in aged mice, CD4+CD25high T cells maintained the ability to suppress the activation of normal young CD4+CD25+ T cells (Fig. 1D), demonstrating that the functional maintenance of CD4+CD25high T cells is independent of the age-related changes of the thymic microenvironment. It is presumable that the expression of Foxp3 in (Fig. 1C, right panel) and/or the self-reactivity of CD4+CD25high T cells (28) are important to maintain the suppressive function throughout aging.

In the young, most suppressive/regulatory CD4 T cells were characterized by the constitutive expression of CD25. Even upon polyclonal T cell stimulation, CD4+CD25+ T cells expressed CD25 at higher levels and more persistently than CD4+CD25– T cell-derived activated T cells (29). As mentioned, we observed that CD4+CD25high T cells from aged mice were Foxp3+ (Fig. 1C, right panel) and suppressive (Fig. 1D). CD4+CD25low T cells from aged mice were a mixture of Foxp3+ and Foxp3– cells (Fig. 1C, left panel), suggesting that these CD4+CD25low T cells contain activated/nonsuppressive T cells. Even in humans, the experiments using human peripheral blood lymphocytes revealed that CD4+CD25high T cells, but not CD4+CD25low T cells, exhibited the characteristics of regulatory cells such as hyporesponsiveness and suppression of proliferation (30). Therefore, a better marker of suppressive/regulatory CD4 T cells may be CD25high. In contrast, the CD4+CD25– T cells from aged mice included Foxp3+ suppressive cells (as CD4+CD25– R123lowCD103+ T cells) (Fig. 4,
A, B, and D). Aged CD4⁺CD25⁺R123lowCD103⁻ T cells also included some Foxp3⁺ cells (Fig. 4D). Taken together, these results demonstrate that Foxp3, but not CD25 nor CD103, is the best marker of suppressive T cells even in aged hosts and suggest the need for a specific cell surface marker of suppressive/regulatory CD4 T cells.

Regarding the relationship between the marker CD25 and suppressive function, it was demonstrated that CD4+CD25⁺ T cells transferred into syngeneic nude mice change the level of CD25 expression, i.e., some CD4⁺CD25⁺ T cells adopt the CD4⁺CD25⁻ phenotype, although they were still Foxp3⁺ and suppressive (16). Furthermore, we have shown that the responsiveness of young CD4⁺CD25⁺ R123low cells (a mixture of R6 and R7) was slightly lower than young CD4⁺CD25⁺ R123high cells (25), although it was not a dramatic hyporesponsiveness like R6 cells and/or R7 cells in aged mice (Fig. 4, B and C), that both young and aged CD4⁺CD25⁺ R123low T cells contained Foxp3⁺ cells (Fig. 4, D and E), and that young CD4⁺CD25⁺ T cells exhibited R123low phenotype (25). Therefore, it is possible that CD4⁺CD25⁺ R123lowCD103⁺ (and/or CD103⁻) Foxp3⁺ T cells in aged mice are derived from CD4⁺CD25⁺ (and/or CD25⁻) Foxp3⁺ suppressive T cells in the young. The relation between young CD4⁺Foxp3⁺ T cells and aged CD4⁺CD25⁺ R123low Foxp3⁺ T cells will need to be investigated further. In addition, although analyses in vitro revealed that aged CD4⁺CD25⁺ R123lowCD103⁺ Foxp3⁺ T cells were anergic and suppressive (Fig. 4B) similar to CD4⁺CD25⁺ T cells in the young, at present, it is not clear whether these aged CD4⁺CD25⁺ R123lowCD103⁺ Foxp3⁺ T cells have a role as immunoregulatory cells to maintain self-tolerance. CD4⁺CD25high T cells maintained their suppressive function even in aged mice (Fig. 1D). Therefore, it is unlikely that, in addition to aged CD4⁺CD25⁺ T cells, aged CD4⁺CD25⁺R123lowCD103⁻Foxp3⁺ T cells were actively required to maintain self-tolerance in aged hosts.

In humans, it is reported that, during the activation of CD4⁺CD25⁺ T cells in an immune response, two populations of cells may arise, effector CD4⁺CD25⁻ cells and Foxp3-expressing regulatory CD4⁺CD25⁺ T cells (31). Therefore, we examined whether the activation of CD4⁺CD25⁺ T cells results in the expression of Foxp3 in aged mice, accounting for the existence of CD4⁺CD25⁺ Foxp3⁺ T cells in aged mice (Figs. 3B and 4D). We purified cells in R7 and R8 in Fig. 4A from aged mice, cultured each cells (1×10⁷/well in 96-round-bottom plate) along with splenic APC (1×10⁴/well), anti-CD3 Ab (5% SN), and IL-2 (10 U/ml). Seven days later, cells were harvested and used for a real-time PCR experiment to evaluate the amount of Foxp3 in aged CD4⁺CD25⁺ T cells before and after an in vitro stimulation culture. However, the activation culture induced no changes in the amount of Foxp3. For example, the relative Foxp3 expression (Foxp3/HPRT) in aged CD4⁺CD25⁺ R123lowCD103⁻ T cells (cells in R7 in Fig. 4A) and CD4⁺CD25⁺ R123high T cells (R8) was 0.15 and 0.11 before stimulation and 0.04 and 0.08 after stimulation, respectively (compared with 7.49 for CD4⁺CD25⁺ R123highCD103⁻ T cells). Therefore, it is likely that this population (CD4⁺CD25⁺ Foxp3⁺ T cells in aged mice) accumulates over time and under specific conditions in vivo, independently of any stimulation.

Age-related defects in CD4 T cell function have been well documented (20, 22). The consequences of these defects are substantial and lead to an increased incidence of infectious diseases in the elderly. Therefore, it is very important to improve the function of CD4 T cells in the elderly. In that regard, the results in this report suggest the importance of studies focused on the function of each CD4 T cell population, not on the whole CD4 T cell population. Our results show that aged CD4 T cells can be divided into suppressive Foxp3⁺ T cells (consisting of CD25⁻ and CD25⁺ T cells), hyporesponsive Foxp3⁺ R123low cells, and normal responsive Foxp3⁺ R123high cells. By inhibiting the suppressive function of Foxp3⁺ cells, by augmenting the responsiveness of normal Foxp3⁺ R123high cells included as a minor population among aged CD4 T cells, or by preventing the gain of low responsiveness in Foxp3⁺ R123low cells as a major population, proper treatments for each CD4 T cell subpopulation, not for CD4 T cells overall, would be needed for improving the CD4 T cell function in aged mice.

Disclosures
The authors have no financial conflict of interest.

References


