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Bim Dictates Naive CD4 T Cell Lifespan and the Development of Age-Associated Functional Defects

Hirotake Tsukamoto,*† Gail E. Huston,*, John Dibble,*, Debra K. Duso,* and Susan L. Swain*

With age, peripheral naive CD4 T cells become both longer lived and functionally impaired and they express reduced levels of Bim, a proapoptotic Bcl family member. In this study, we show that reduced Bim expression by naive CD4 T cells intrinsically mediates their longer lifespan in the periphery. Moreover, using mixed bone marrow chimeras reconstituted with Bim+ and Bim− bone marrow cells, Bim− naive CD4 T cells exhibit accelerated development of age-associated dysfunctions, including reduced proliferation and IL-2 production and defective helper function for B cells, without any increase in their turnover. However, newly generated Bim− naive CD4 T cells in middle-aged mice are not defective, indicating an additional requirement for their persistence in the periphery. These age-associated immune defects develop independently of the “aged” host environment and without extensive division, distinguishing them from classic “senescence.” We suggest that the reduction of Bim levels with age in naive CD4 T cell is the initiating step that leads to increased cellular lifespan and development of age-associated functional defects. The Journal of Immunology, 2010, 185: 4535–4544.

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ger-related changes in T cell function lead to progressive defects in the ability of aged individuals to mount protective immune responses, which can increase their susceptibility to emerging cancers and bacterial and viral infections, and decrease effectiveness of vaccines (1, 2). Hence, it is critical to understand the defining mechanisms that lead to the impaired immune function of T cells for developing therapies and increasing vaccine efficacy. In mice, similar immune defects also develop progressively with age and, as in humans, naive T cells develop defective T cell memory and reduced responsiveness to vaccines, providing a model system in which to analyze the mechanisms leading to age-associated T cell defects (1).

Despite the drastic decrease in output of T cells from the thymus in aged animals, the total number of peripheral T cells does not fall precipitously over an animal’s lifetime (3–5). It has been suggested that the oligoclonal expansion of CD4+ memory phenotype T cells that occurs with aging provides a feedback mechanism to compensate for the reduced thymic output of T cells (6, 7). Indeed, such clonal expansion, which occurs more prominently among CD8 T cells than CD4 T cells, results in a skewed TCR repertoire that can lead to defective responses to infection (8). However, previous studies suggest that aged naive CD4 T cells (9–11) have more pronounced immune defects than CD8 T cells (12) and that memory CD4 T cells generated in early life do not develop defects as quickly (13). Thus, the decreases in CD4 T cell-mediated responses in the aged are more likely to be due to a decline in per cell function of naive CD4 T cells. However, it remains unclear how the aging process leads to the striking functional defects observed in the naive CD4 T cell population.

T cell homeostasis is achieved by a tightly regulated balance of cell division and death. Peripheral naive CD4 T cells in young mice have a short lifespan (14) and soon undergo apoptosis, creating a steady state in which newly generated thymic emigrants make up the loss as the peripheral cells expire (1, 15, 16). Once naive T cells are activated in response to Ags, they divide many times and mature into effectors. Most of the effectors undergo apoptosis following Ag clearance, resulting in the dramatic contraction of the population followed by the transition of the remaining cells to a memory state. The apoptosis of activated T cells can be initiated through surface death receptors, such as Fas-Fas ligand and TNFR family members (16, 17), although it can also occur because of withdrawal of growth and survival factors. Apoptosis of resting cells such as naive CD4 T cells occurs mostly when cell-intrinsic pathways are activated by diverse stresses such as cytokine and serum withdrawal, DNA damage, or steroid exposure (18). Bcl family members are key regulators of these intrinsic pathways. Bcl-2 and Bcl-xL exert antiapoptotic actions that are blocked when they bind to proapoptotic molecules such as Bim. BH3-only proteins, including Bim, activate executioner molecules, such as Bax (18, 19), resulting in apoptosis. Several studies suggest that the levels of Bim determine the extent of T cell survival under conditions of limited survival cytokines, exposure to reactive oxidative species, and DNA damage (20–22). Bim-deficient mice have 2- to 5-fold more CD4 and CD8 T cells, compared with wild-type (WT) mice, likely reflecting both impaired negative selection of thymocytes (23) and increased resistance to spontaneous apoptosis of T cells in the periphery (15, 20). Thus, there is good evidence that Bim regulates multiple aspects of T cell homeostasis in situ.

Our previous studies suggested that the development of aging defects in naive CD4 T cells is dependent on their persistence in the periphery (9, 14). We considered the possibility that if naive CD4 T cells in the periphery become longer lived with age, their...
extended lifespan could be responsible for their development of age-associated functional defects. Indeed, our and other recent studies demonstrated that naive CD4 T cell lifespan becomes progressively longer with age (11, 14, 24), and that the increased longevity correlated with an increased resistance to spontaneous apoptosis of aged naive CD4 T cells (14, 24–26).

We found a correlation between longer lifespan of aged naive CD4 T cells and decreased levels of Bim expression with age (Ref. 14 and this paper), suggesting that reduction in Bim levels might dictate the longer lifespan by causing increased resistance to spontaneous apoptosis. Moreover, because the development of functional defects in naive CD4 T cells depends on their persistence in the periphery, we postulate that Bim reduction might facilitate the generation of defects by increasing their lifespan. To test this hypothesis we generated mixed bone marrow (BM) chimeras reconstituted with both WT Bim+/+ and heterozygous Bim KO (Bim+/−) BM cells. We find in this study that cell-intrinsic reduction of Bim expression is sufficient to induce both longer lifespan and accelerated development of age-related functional defects in naive CD4 T cells from middle-aged BM chimeras. Our results provide strong evidence that Bim levels are a major determinant of naive CD4 T cell lifespan and secondarily of the development of age-associated functional defects.

**Materials and Methods**

**Mice**

Bim-deficient mice were originally provided by A. Strasser (Walter and Eliza Hall Institute, Melbourne, Australia) to R. Budd (University of Vermont, Burlington, VT). C57BL/6 background, bim heterozygous and homozygous Thy1.1+ AND TCR transgenic (Tg) mice were bred and housed at the Trudeau Institute Animal Facility. All mice including CD45.1+ B6.SJL-PtprcaPep3b/BoyJ mice and CD4 KO mice were fed standard sterile diet ad libitum and were housed in isolator cages under specific pathogen-free conditions. CD45.2 and biotinylated anti-CD45.1 Abs (eBioscience, San Diego, CA) were purchased from SouthernBiotech (Birmingham, AL).

**Flow cytometry**

The following Abs were used: FITC-labeled anti-CD44 or anti-mouse IgG1; PE-labeled anti-Vβ3, anti-CD38, anti–Bcl-2, or anti-mouse IgM; PerCP-labeled anti-CD4 or anti-B220; PE-Cy7–labeled anti-Thy1.2; allophycocyanin-anti-Thy1.1 (all purchased from BD Pharmingen, San Diego, CA); Alexa Fluor 405-labeled anti-CD44 (Caltag Laboratories, Burlingame, CA); allophycocyanin-Alexa Fluor 750-labeled anti-CD45.2 and biotinylated anti-CD45.1 Abs (eBioscience, San Diego, CA) combined with Pacific Orange-streptavidin development (Invitrogen, Carlsbad, CA). Biotin-pseudoglutamin (PNA) was purchased from Invitrogen. 4-Hydroxy-3-nitrophenyl acetyl (NP)-conjugated allopseudohcycyanin was prepared and stained as described previously (27). Polyclonal anti-Bim Ab was from Cell Signaling Technology (Danvers, MA). Seven-color immunofluorescence was analyzed by using a FACSCanto II cytometer (BD Biosciences, San Jose, CA).

Following in vitro stimulation with or without cognate Ag, cultured cells were stained for surface markers, fixed using a 2% paraformaldehyde solution at room temperature for 10 min, permeabilized with saponin, and then stained with Pacific Blue-conjugated anti–IL-2 Ab (eBioscience, San Diego, CA). Intracellular Bim and Bcl-2 proteins were monitored with flow cytometry as described previously (14). Data files were analyzed by using FlowJo software (Tree Star, Ashland, OR).

**Adoptive transfer and immunization**

Naive CD4 T cells from pooled spleen and lymph were isolated as previously described (14), or Bim+/+CD45.1+ and CD45.1− Bim+/− or Bim+/−/Vb3− CD44+ naive cells were sorted by FACS Vantage SE flow cytometer equipped with the DIVA digital processing system (BD Immunocytometry Systems, San Jose, CA). Purified naive CD4 T cells were injected i.v. into host mice. Mice were immunized i.p., with 200 μg NP-conjugated pigeon cytochrome c (PCC) or PBS in alun.

**Proliferation assays**

Sorted naive AND TCR Tg T cells (2.5 × 10^6/well) were stimulated with irradiated DCEK-ICAM/B7 cells (2.5 × 10^5/well) and indicated concentration of PCC peptide in 96-well culture plates. After 60 h of coculture, T cells were pulsed with ^[3]H]thymidine, and the incorporation of radiolabel was determined by counting radioactivity 18 h later. For measurement of IL-2 secretion, supernatants collected 48 h after stimulation with Ag were assayed in a bioassay using the IL-2–sensitive NK-3 cell line.

**ELISA**

Serum was collected from immunized animals, NP-specific IgG1 was determined by ELISA with NP_{4}_{4}_{4}_{4}_{4} conjugated BSA (27). HRP-conjugated goat anti-mouse IgG1 Abs were purchased from SouthernBiotech (Birmingham, AL).

**Real-time RT-PCR**

Total RNA was extracted from sorted naive CD4 T cells using an E.Z.N.A. Total RNA kit (Omega Bio-Tek, Norcross, GA). Contaminating DNA was removed using a DNA-free kit (Ambion, Austin, TX). First-strand cDNA was synthesized using cDNA reverse transcription kits (Applied Biosystems, Foster City, CA). TaqMan probe for p19 alternative reading frame (p19ARF; Mm01257348, INK4a (Mm00494449), and INK4b (Mm00483241) were obtained from Applied Biosystems. The primers and probes for GAPDH designed by Dr. S. Smiley and P.S. Adams (Trudeau Institute, Saranac Lake, NY) were used. All samples were analyzed in triplicate using an ABI Prism 7700 sequence detection system (Applied Biosystems) and were normalized with GAPDH expression. The threshold cycle (C_{T}) values for each reaction were determined and averaged using TaqMan SDS analysis software. The changes in gene expression were calculated by the comparative C_{T} method (fold changes = 2^{Δ^-ΔC_{T}}).

**Statistical analysis**

All statistical analyses were performed using Prism 4.0 software (GraphPad Software, San Diego CA). Differences were determined by an unpaired two-tailed Student t test, assuming unequal variance. In the case of comparison of T cells from mixed BM chimeras, a paired t test was performed for statistical analysis. The p values <0.05 were considered significant. The regression lines and their slopes were determined by plotting data on logarithmic scale and performing linear regression analysis.

**Results**

**Inverse correlation between Bim expression and in vivo lifespan of naive CD4 T cells**

In previous studies using a TCR Tg mouse model, we found that naive CD4 T cell lifespan was inversely correlated with expression of Bim. To evaluate whether longevity also progressively increased among non-Tg naive CD4 T cells, we examined the intrinsic lifespan of polyclonal naive CD4 T cells derived from mice of different ages by transferring them to normal young lymphopereote hosts and assessing their survival in the periphery. At 30 d after transfer, we found an age-dependent increase in the ability of transferred donor T cells to survive that was proportional to donor age (Fig. 1A). This increase in persistence in vivo of donor-aged naive CD4 T cells was not due to an alteration in migration since, in cotransfer experiments, aged T cells outnumbered young T cells in each organ tested (Supplemental Fig. 1). Consistent with previous results, TCR-mediated proliferation decreased progressively with donor age and was pronounced even when naive CD4 T cells were stimulated with the strong stimulus of anti-CD3 and CD28 Ab (Fig. 1B).

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Moreover, we found that the progressive age-dependent decrease in Bim expression correlated well with the increase of lifespan of naive CD4^+ T cells (Fig. 1C). The balance of Bim and Bcl-2 proteins is critical for determining cell survival rate (15, 28). Therefore, we also examined the expression of Bcl-2 in aged naive CD4 T cells. As previously reported (24), Bcl-2 expression also decreased with aging (Fig. 1D). This could be due to a mutual control of Bcl-2 and Bim expression in T cells (29). However, the degree was smaller than that of Bim reduction, and the ratio between Bim/Bcl-2 in aged T cells was lower than that in young cells (Fig. 1E, 1F). These results support the concept that reduced Bim expression is directly responsible for the increase in lifespan of aged naive CD4 T cells.

**Bim determines the in vivo lifespan of naive CD4 T cells**

To further assess how levels of Bim expression correlate with increased in vivo lifespan of naive CD4 T cells, Bim^+/+, Bim^−/−, and Bim−/− AND TCR Tg mice were generated. The Bim expression in young Bim−/− T cells was similar to the levels observed in aged naive CD4 T cells (Fig. 2A). We monitored the number of donor naive CD4 T cells from each mouse after transfer into young hosts. As shown in Fig. 2B, the lifespan of naive CD4 T cells increased as the level of Bim expression decreased. The increased longevity of T cells with reduced Bim expression was not due to higher IL-7R expression because IL-7R expression was comparable among Bim^+/+, Bim^−/−, and Bim^+/− T cells, and T cells with reduced Bim expression were refractory to apoptosis in the presence or absence of IL-7 (Ref. 14 and data not shown). When we prelabeled donor cells with CFSE and examined the CFSE intensity of each population. There was no appreciable division of donor cells regardless of the level of Bim expression. Thus, differences in division rates were not responsible for the different rate of cell persistence. Moreover, when Bim^+/+ WT and Bim^−/− naive CD4 T cells were transferred into ATxBM mice, where homeostatic proliferation is induced by the absence of host T cells (30), we found similar CFSE profiles for both donor WT and Bim^−/− T cells at 10 d after transfer (Fig. 2C). This indicates that reduced levels of Bim expression did not directly impact the rate of homeostatic proliferation. The significant increase in donor T cell recovery found in the populations expressing reduced Bim in the lymphopenic environment are also consistent with the ability of Bim-deficient naive CD4 T cells to resist spontaneous apoptosis (Fig. 2D).

We next investigated the kinetics of decay of naive CD4 T cells expressing different levels of Bim in situ. For this purpose, we generated mixed BM chimeras reconstituted with donor BM cells from Bim^+/+ and Bim^−/− AND TCR Tg mice. At 2 mo after BM transfer, these mixed BM chimeras were thymectomized to prevent additional de novo production of naive T cells. As shown in Fig. 2E, we transferred a smaller number of Bim^+/− BM cells into the host mice so that the numbers of reconstituted Bim^+/− Tg^+ naive T cells were initially lower than those of WT Tg^+ T cells in pretthymectomized BM chimeras. After thymectomy, the number of WT naive T cells rapidly decreased, whereas Bim^+/− T cells declined more slowly. By 20 d postthymectomy, the number of resident Bim^+/− naive CD4 T cells was significantly higher than that of WT cells (Fig. 2E). In this setting, we also analyzed the in vivo turnover of peripheral naive T cells by giving BrdU to the chimeras. As shown in Fig. 2F, the fraction of divided BrdU^+ naive T cells was small in the thymectomized mice in both WT and Bim^+/− naive populations, whereas it was high among memory phenotype T cells (more than half) in both groups after 7 and 14 d of labeling. Collectively, these results provide strong evidence that reduced Bim expression results in a dramatic, cell-intrinsic prolongation of naive CD4 T cell lifespan, but has no discernible effects on their homeostatic division in the periphery.
The development of age-related functional defects in naive CD4 T cells is accelerated in Bim−/− mice

Because development of age-associated functional defects in naive CD4 T cells is dependent on their increased persistence in the periphery (9, 11, 14), and because naive CD4 T cells with reduced Bim have increased longevity in vivo, we postulated that the reduction of Bim in naive CD4 T cells that occurs with age might be required for their development of age-related defects. Alternatively, the defects and Bim reduction might occur concurrently, but independently. To evaluate these hypotheses, we generated aged Bim−/− AND Tg mice and determined the number and function of Bim−/− naive CD4 T cells from youth to middle age. Consistent with previous studies using non-TCR Tg mice (23), the number of CD4+CD8− single-positive thymocytes was significantly higher in young Bim−/− AND TCR Tg mice than that in WT mice (Supplemental Fig. 2). This is likely because of decreased apoptosis of thymocytes in the cells with reduced Bim expression (23). Additionally, the numbers of both peripheral CD4 T cells and Tg+ naive CD4 T cells were also increased by Bim heterozygosity in both young and middle-aged mice. In both Bim+/− and Bim−/− mice, there was an age-dependent decrease in the numbers of total thymocytes and peripheral Tg+ T cells, but the numbers in aged Bim−/− mice were higher than in age-matched WT mice.

To evaluate the effect of Bim reduction on the development of age-related functional defects, naive CD4 Tg+ T cells from Bim+/− and Bim−/− AND Tg mice of different ages were stimulated with cognate Ag, PCC peptide-pulsed APCs. Naive Tg+ T cells from 2-mo-old Bim−/− mice proliferated at a rate comparable to that of
young WT T cells (Fig. 3A), confirming that reduced Bim expression did not directly, in and of itself, impair proliferation. However, naive CD4 T cells from Bim<sup>−/−</sup> mice 6 mo of age proliferated significantly less than did those from age-matched WT mice, which did not yet show an age-related decrease in proliferation. By 9 mo of age, there was also a slight decrease in the proliferation of WT population so that the difference between WT and Bim<sup>−/−</sup> T cells from 9 mo-old mice was less dramatic than that observed in T cells from 6 mo-old mice. Importantly, Bim<sup>−/−</sup> Tg naive T cells from 6-mo-old, but not 2-mo-old, mice also exhibited a significant age-related decrease in IL-2 production in response to Ag recognition (Fig. 3B). These results support the hypothesis that increased longevity of naive CD4 T cells is caused directly by a reduction of Bim expression, and that longer persistence due to the increased lifespan enables them to develop age-related functional defects more quickly. Thus, both reduced Bim and the passage of time are required for the development of the standard functional defects in division and IL-2 production.

**Bim reduction causes a cell-intrinsic accelerated development of age-related immune defects in naive CD4 T cells**

Given that Bim is expressed not only by T cells but also by other cell lineages including other hematopoietic cells, epithelial, neuronal, and germ cells (31), the environment surrounding T cells could be altered in Bim<sup>−/−</sup> mice, and thus T cell-extrinsic differences could potentially explain the differences in naive CD4 T cell responses. Therefore, we generated mixed BM chimeras by reconstituting lethally irradiated hosts with a mixture of BM cells from both WT and Bim<sup>−/−</sup> AND Tg mice (Supplemental Fig. 3). These mice were then left to mature to middle age, which should allow defects sufficient time to develop in the longer lived cells. WT and Bim<sup>−/−</sup> donor naive CD4 T cell populations were both produced and aged in the same chimeras, allowing us to exclude T cell-extrinsic effects of Bim. As shown in Fig. 4A, the numbers of Bim<sup>−/−</sup> Tg<sup>+</sup> single-positive thymocytes declined progressively with age in the mixed BM chimeras, and the decay was almost the same as that of WT counterparts. At 3 mo of age, the numbers of Bim<sup>−/−</sup> peripheral naive Tg<sup>+</sup> CD4 T cells was slightly lower than those of WT cells in the mixed BM chimeras because of a smaller number of transferred Bim<sup>−/−</sup> BM cells (Fig. 3E, 4B). However, the rate of age-dependent progressive loss of the Bim<sup>−/−</sup> naive Tg<sup>+</sup> T cell cohort was significantly lower than that of WT cells (Fig. 4B), suggesting increased persistence of Bim<sup>−/−</sup> naive CD4 T cells in the periphery. These data provide strong support for the concept that the effects of reduced Bim expression are cell intrinsic.

We next analyzed the function of WT and Bim<sup>−/−</sup> naive T cells isolated from the mixed BM chimeras by cell sorting and tested the ability to respond to Ag ex vivo. Both WT and Bim<sup>−/−</sup> naive CD4 T cells from 3-mo-old chimeras divided at the same rate and produced equivalent levels of IL-2 (Fig. 4C, 4D). At 6.5 mo there was little evidence of age-related defects in proliferation or IL-2 production of the WT BM-derived naive CD4 T cells. In contrast, the Bim<sup>−/−</sup> naive T cells from the 6.5-mo-old middle-aged BM chimeras gave significantly reduced responses, and the defects were also notable at 10.5 mo. The acceleration of age-related functional defects in proliferation and IL-2 production associated with reduced Bim were also observed at the single-cell level since nearly 5-fold fewer Bim<sup>−/−</sup> cells produced intracellular IL-2 (Fig. 4E), and there was less division measured by loss of CFSE after Ag stimulation (Fig. 4F). Furthermore, as observed previously in physiologically aged naive CD4 T cells (9, 14), the defect in proliferation of middle-aged Bim<sup>−/−</sup> naive T cells, seen at 6.5 mo, could be restored by the addition of exogenous IL-2 (Fig. 4F). This is consistent with the hypothesis that reduced IL-2 production in middle-aged Bim<sup>−/−</sup> naive T cells is largely responsible for their blunted proliferation.

To further evaluate the correlation between the development of functional defects and degree of reduced Bim expression, we examined the TCR-mediated response of WT, Bim<sup>−/−</sup>, and Bim<sup>−/−</sup> naive CD4 T cells from 7-mo-old middle-aged BM chimeras. As shown in Fig. 4G, the middle-aged homozygous KO (Bim<sup>−/−</sup>) T cells produced less IL-2 than did Bim<sup>−/−</sup> heterozygous T cells and much less than did WT cells. Thus, the extent of the functional defects was inversely correlated with the level of Bim expression. A similar hierarchy was observed in proliferative responses (Supplemental Fig. 4A).

To confirm that the defects induced by reduced Bim only develop during peripheral persistence of naive CD4 T cells, we analyzed the responses of newly generated naive CD4 T cells from the thymus of the middle-aged chimeras. To promote the production of new CD4 T cells in situ, we depleted the existing peripheral CD4 T cells in 6-mo-old mixed BM chimeras by administering Ab to CD4. The treatment efficiently depleted the host Tg<sup>+</sup> T cells, and we detected new Tg<sup>+</sup> CD4 T cells reconstituting the population during 3 wk (Supplemental Fig. 4B). Similar kinetics of reconstitution were seen for both WT and Bim<sup>−/−</sup> Tg<sup>+</sup> T cells. One month after CD4 T cell depletion, we harvested the newly generated Bim<sup>−/−</sup> and Bim<sup>−/−</sup> naive CD4 T cells and stimulated them with cognate Ag. In control animals with no depletion, the Bim<sup>−/−</sup> cohort proliferated less, as we expected, but this defect was restored in the newly generated Bim<sup>−/−</sup> T cells in middle-aged BM chimeras after CD4 T cell depletion (Fig. 4H and Supplemental Fig. 4C).

**Bim<sup>−/−</sup> T cells develop reduced in vivo helper function by middle age**

A key age-associated defect of naive CD4 T cells is a profound decrease in the helper activity that is needed to promote cognate B cell responses in germinal centers, resulting in poor production of isotype-switched specific Ab (11, 27). To test whether this key activity decays more rapidly in Bim<sup>−/−</sup> naive CD4 T cells, we transferred Bim<sup>−/−</sup> or Bim<sup>−/−</sup> naive AND T cells that were purified from 7-mo-old mixed BM chimeras and were CFSE-labeled into T cell-deficient ATxBM mice and then immunized them with the

![Figure 3](http://www.jimmunol.org/Downloaded-from/10.4049.jimmunol.1600237/Figure3.jpg)
FIGURE 4. Aged Bim<sup>−/−</sup> naive CD4 T cells develop accelerated functional defects in mixed BM chimeras. A, Changes with age in the number of CD4<sup>+</sup>CD8<sup>−</sup> Tg<sub>+</sub> thymocytes in mixed BM chimeras are shown. There is no significant difference between the slopes of linear regression lines shown here (Bim<sup>+/+</sup> versus Bim<sup>−/−</sup>) was statistically significant. C and D, The proliferative response (C) and IL-2 production (D) of Bim<sup>+/+</sup> and Bim<sup>−/−</sup> naive AND Tg CD4 T cells to Ag were assessed at different ages. Naïve cells were sorted from mixed BM chimeras 1.5, 5, and 9 mo after BM reconstitution, and then they were stimulated with 0, 1, and 5 μM antigenic peptide-pulsed APCs. Results are presented as the mean ± SEM of triplicate assay. E, Bim<sup>+/+</sup> and Bim<sup>−/−</sup> naive AND Tg cells were isolated from BM chimeras 6 mo after reconstitution. IL-2 production in CD4<sup>+</sup>Vβ3<sup>+</sup> populations in response to cognate Ag was determined by intracellular cytokine staining. F, Naïve AND T cells as described in E were stimulated with or without cognate Ag and with and without addition of 10 ng/ml IL-2. Four days later, dilution of CFSE was determined. G, IL-2 production from indicated naïve CD4 T cells from mixed BM chimeras is shown. At 7 mo after BM reconstitution, naïve AND Tg cells isolated from mixed chimeras were stimulated as described in C. H, Proliferation of newly generated naïve CD4 T cells is shown. Six-month-old mixed BM chimeras were treated with anti-CD4 or isotype matched control Ab (200 μg). One month later, naïve AND Tg<sup>+</sup> cells from the chimeras were stimulated as described in C. Similar results were obtained from at least two independent experiments. *p < 0.05; **p < 0.01. nd, not detected.
have developed a reduced ability to provide the help for cognate B cells and that the age-associated defects in the B cell helper activity of naive T cells develop at an accelerated pace when Bim is reduced.

Collectively, the results in mixed BM chimeras argue strongly against the possibility that the age-related functional changes that are accelerated by reduced Bim expression are due to any extrinsic effects of Bim deficiency on the host mice. Instead, these results suggest that it is T cell-autonomous reduction of Bim expression that facilitates the accumulation of long-lived, dysfunctional naive CD4 T cells in the periphery with the passage of time.

Increased expression of senescence-related genes in long-lived naive CD4 T cells

Age-related functional declines in the regenerative capacity of many tissues and the loss of ability of cells to proliferate are often correlated with increased expression of INK family tumor suppressor genes, which block cell cycle progression by inhibiting the activity of cyclin-dependent kinases 4 and 6, or which inhibit cell cycle by activating the p53 tumor suppressor (32, 33). It has been suggested that expression of these genes could serve as a biomarker of cellular senescence (34, 35). Therefore, we examined whether these genes were upregulated in naive CD4 T cells with age and whether Bim deficiency would lead to their early expression in longer lived, functionally impaired middle-aged CD4 T cells. Naive CD4 T cells from 20-mo-old AND TCR Tg mice expressed significantly higher levels of INK4a, INK4b, and p19ARF mRNA than did equivalent young naive CD4 T cells (Fig. 6A). Similar upregulation was seen in polyclonal naive CD4 T cells from elderly mice (H. Tsukamoto and S.L. Swain, unpublished data). However, increased expression of INK4a, INK4b, and p19ARF was reported previously in hematopoietic stem cells from aged mice (34, 36, 37), so it is possible that the upregulated expression of these genes might be independent of peripheral cellular aging. To explore this possibility, we examined the expression of INK family tumor suppressors in the newly generated naive CD4 T cells derived from aged hematopoietic stem cells by isolating naive CD4 T cells from BM chimeras reconstituted with aged or young AND BM cells. The expression of INK4a and p19ARF, but not INK4b, in T cells differentiated from aged and young BM cells was comparable, indicating that newly generated naive CD4 T cells derived from aged hematopoietic stem cells did not express higher levels of mRNA for INK4a and p19ARF (Fig. 6A). Naive CD4 T cells from the BM chimeras expressed lower levels than did naive cells from untreated aged mice, but higher levels than in young cells, perhaps due to the effect of irradiation that was used to generate the BM chimeras and is known to increase expression of these genes (38).
Newly generated naive AND T cells were isolated from BM chimeras reconstituted with BM cells from young or aged AND Tg mice described above.

Young, aged, and newly generated naive T cells are shown. Naive AND T cells purified from 2- (young) or 18-mo-old (aged) AND Tg mice are shown (so by increasing cellular lifespan since that is responsible expression of senescent markers. One likely possibility is that it does expression, similar to functional defects in naive CD4 T cells, in-

To further test the relationship of expression of INK4a and p19ARF to cellular persistence, we determined whether their expression would be detected earlier in life in the longer lived Bim+/−naive CD4 T cells. Indeed, we found significant upregulation of INK4a and p19ARF but not INK4b in the Bim+/−naive CD4 T cells derived from 10-mo-old mixed BM chimeras but not 2.5-mo-old mice. These results suggest that INK4a and p19ARF expression, similar to functional defects in naive CD4 T cells, increase with greater cellular age or persistence. Thus, reduced Bim expression acts in a cell-intrinsic manner to cause accelerated expression of senescent markers. One likely possibility is that it does so by increasing cellular senescence since that is what is responsible for accelerating development of functional defects.

**Discussion**

Our previous studies indicated that the main mechanism that accounts for the persistence of the peripheral naive CD4 T cell population with advanced age is that the cellular lifespan becomes progressively longer without the cells undergoing more homeostatic division (14). In the naive CD4 T cell population the increased longevity is correlated with loss of immune function and with reduction in expression of Bim. In this study, we show evidence that a reduction in Bim expression is sufficient to directly regulate a cell-intrinsic increase in naive CD4 T cell lifespan, supporting the hypothesis that Bim levels play an essential role in naive CD4 T cell homeostasis (15, 20). We show in this study that the reduced Bim-mediated increase in the lifespan of naive CD4 T cells combined with the passage of time causes the development of age-associated immune defects in the naive CD4 T cells, including their attenuated proliferation, impaired IL-2 production, and reduced helper activity for B cells, as well as increased expression of senescence-associated markers. We suggest the reduction of Bim expression is a first step that eventually drives the loss of immune function in aged naive CD4 T cells.

T cell homeostasis maintains the number of T cells in each of multiple subsets such as CD4 and CD8 and naive and memory subsets of each that are necessary for optimal immune responses to new and previously encountered pathogens. We suggest that the high levels of Bim expressed in young naive CD4 T cells dictates a short lifespan and thus facilitates the rapid turnover of the naive CD4 T cell pool so that naive T cells are continually depleted from the periphery and replaced by newly generated ones. Extensive thymic involution and reduction in thymopoiesis and export of new naive T cells into the peripheral pool occur with aging. Despite these phenomena, a substantial naive CD4 T cell population is retained in aged mice (4, 5). We find that reduced Bim expression with age drives the accumulation of peripheral naive CD4 T cells by increasing their lifespan but not their turnover, implying that reduced Bim expression contributes to the maintenance of this population in aged mice. However, the slower turnover of naive CD4 T cells with age as mediated by decreased thymic output, as well as the reduced Bim-induced longer T cell persistence that we have described, should lead to a smaller available TCR repertoire and could thus contribute to the poor responsiveness of the elderly as observed in the constricted CD8 T cell repertoire in aged mice, leading to less efficient response to influenza virus (8). Given these considerations, we suggest that the depletion of dysfunctional aged peripheral naive CD4 T cells and their reconstitution by newly generated ones would be one approach that could potentially restore the immune function of CD4 T cells in aged animals.

The mechanisms underlying the downregulation of Bim in aged naive CD4 T cells are not yet known. It has been demonstrated that Bim expression is regulated by Foxo3a and Runx3 at the transcriptional level (39, 40) and by ERK at the posttranscriptional level (41), respectively. We favor the possibility that in aged naive CD4 T cells, the reduction of Bim expression may be posttranscriptionally regulated since mRNA expression for Bim is comparable in young and aged naive CD4 T cells (H. Tsukamoto and S.L. Swain, unpublished data). However, there was no obvious difference in ERK-mediated Bim phosphorylation between young and aged T cells (data not shown). A detailed understanding of regulatory mechanisms for reduced Bim expression in aged naive CD4 T cells may be applicable to the approach to delete Bim reduced, dysfunctional aged naive CD4 T cells, although we have not yet examined whether forced expression of Bim in aged naive CD4 T cells can restore a susceptibility to apoptosis. These issues are under investigation.

In the cohort of naive CD4 T cells in mixed BM chimeras with reduced Bim, age-associated defects comparable to those of WT cells from animals 10 mo or older are found reproducibly by 6–7 mo of age, indicating that the development of functional defects were mostly independent of those changes in the T cell-extrinsic host environment that only occur with advanced age. In support of this idea, we showed recently that adult thymectomy, which also leads to a population of peripheral naive CD4 T cells of greater cellular age, leads to a similar accelerated development of...
immune defects even in middle-aged mice (14), and, conversely, that in mice where superantigen deletion prevents accumulation of aged naïve CD4 T cells, many age-associated defects do not develop even when the mice are elderly (11). Collectively, these results suggest that the first effect is a reduction of Bim levels that acts to increase lifespan and that this in turn leads to an increase in the cellular age of the population of T cells and the concomitant development of functional defects. We therefore suggest that the age of the naïve CD4 T cell is proportional to the degree of functional defects and that Bim is a biomarker for both.

Several other models support an inverse correlation between increased or decreased susceptibility to apoptosis in T cells and development of defects in proliferation. Mountz and colleagues (26) found a slower age-associated reduction in T cell proliferation in apoptosis-prone CD2-Fas Tg mice. In contrast, it was reported that T cells from p53-deficient mice that are refractory to spontaneous apoptosis exhibit enhanced age-associated defects in proliferation (42). Although the published studies did not rule out the possibility that T cell-extrinsic factors could be responsible for the altered phenotypes, a causal link between reduced function with age in T cells and their longevity in the periphery may play a role in these other models.

Age-associated defects of naïve CD4 T cells are responsible for poor CD4 memory T cell generation (13) and the loss of efficient cognate help with age (27). These lead to the poor efficiency of successful primary vaccination in aged mice, and it is likely that similar deficiencies in humans could be responsible for their poor immune response to newly emerging pathogens and new vaccines (1, 2). The loss of helper function was accelerated in naïve CD4 T cells with reduced Bim (Fig. 5) and was T cell intrinsic. Helper defects were unlikely to be a direct consequence of reduced proliferation because the absolute number of Bim+ naïve T cells caught up with that of Bim−/− T cells by day 12 after immunization (Supplemental Fig. 5), probably because of their attenuated contraction (17). Attenuated activation of aged CD4 T cells with reduced Bim expression might be due to insufficient Ca2+ influx that was associated with increased Bcl-2 expression (43). However, this explanation is not reasonable because increased Ca2+ influx induced with PMA/sodium ionomycin cannot restore the defective IL-2 production in aged T cells (14), and aged naïve CD4 T cells exhibited decreased, rather than increased, expression of Bcl-2 (Fig. 1). It remains to be determined whether the various functional defects that develop with naïve CD4 T cell aging are all due to one central defect such as defective IL-2 production, or whether the process of cellular aging induces multiple defects in several pathways.

Many cell types in addition to T cells develop proliferative defects with age (44). Many researchers have championed the concept that excessive division is associated with the acquisition of replicative senescence that is usually characterized as a state of permanent growth arrest (32). They have found a correlation between senescence and high expression of INK family tumor suppressor genes p16INK4a and p19ARF whose products impede cell cycle progression (33, 34). In this study, we report that long-lived naïve CD4 T cells, either those isolated from aged mice or those expressing reduced Bim isolated from middle-aged mice, expressed increased levels of mRNA for INK4a and p19ARF, whereas newly generated naïve CD4 T cells derived from aged BM expressed lower levels than did long-lived cells. INK family expression in young Bim−/− and WT cells was equivalent, but INK4a and p19ARF increased more quickly with age in the Bim−/− cells. Thus, their expression is also correlated with cellular age in naïve CD4 T cells.

There are some key differences between classically defined replicative senescence and the loss of function in naïve T cells with aging. First, whereas replicative senescence is caused by extensive proliferation (32), naïve CD4 T cells divide rarely in the periphery and as they age, division is, if anything, diminished (Ref. 14 and Fig. 2C, 2F). However, it is during their peripheral persistence, when naïve CD4 T cells are quiescent and nondividing, that INK4a and p19ARF expression increases and the whole set of immune defects develop, including the reduced ability to proliferate to Ag. Second, although aged naïve CD4 T cells respond less vigorously to antigenic stimulation, they do not exhibit permanent growth arrest, a characteristic of replicative senescent cells (32, 33). Indeed, the presence of exogenous IL-2 or proinflammatory mediators can restore proliferation, expansion, effector generation, and helper function of the naïve CD4 T cells from aged mice (14, 45, 46) and the long-lived cells from middle aged Bim−/− T cells (Fig. 4). Thus, we suggest that the erosion in function that occurs in the naïve CD4 T cell population with cellular age is not comparable to replicative senescence, but to a unique defective state associated with resting cells, and that it is thus likely that mechanisms responsible for the two phenomena are distinct. This suggests that the INK family, and by inference the p53 family pathways, may act not only in traditional senescence and tumor suppressor mechanisms in proliferating cells, but also to reduce responsiveness to external signals in cells that have not undergone division for some time.

The most commonly suggested mechanism to explain age-associated development of defects at the cellular level is that excessive stresses increase and accumulate with age and lead to cellular damage and chromosomal aberrations (47). Potential sources of cellular stress known to undermine cell function include misfolded proteins, oxidative damage, and disrupted chromatin (48–51). Indeed, it has been suggested that the Bim protein serves as a “molecular switch” to induce apoptosis in response to such diverse stresses (20–22, 51). Thus, reduced Bim expression in T cells could interfere with stress-induced apoptosis of T cells and remove the selective process that weeds out damaged cells, allowing defective cells to survive. Our studies in this paper suggest a reconsideration of this viewpoint. First, the lack of dependence of the age-associated changes on host age argues that the factors responsible must be constitutive or that events are programmed and independent of extrinsic factors. One possible explanation is that the long persistence of naïve cells at rest in a state of low metabolism could somehow trigger defects. Further studies are needed to evaluate these hypotheses.

In summary, our results indicate that the level of Bim expression plays a critical, previously unappreciated role in homeostasis and immune competence of naïve CD4 T cells, determining whether they will be eliminated or become longer lived cells that progressively decrease in ability to respond. Because thymic involution and its homeostatic consequences are unique to T cells and correlated with Bim reduction, it is tempting to speculate that one or both of these might lead to Bim reduction with age and increased lifespan, and thus set in motion the progressive loss of function in the naïve CD4 T cell population.

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Disclosures
The authors have no financial conflicts of interest.

References


Supp. Figure 1
Increased persistence of aged naïve CD4 T cells in multiple tissues. A and B, CD45.2+ naïve CD4 T cells (2 x 10⁶) purified from 2-mo and 24-mo old C57BL/6 mice were transferred into young CD45.1+ mice. Thirty days later, cells were harvested from indicated tissues. The profiles of Thy1.2 and Thy1.1 expression in CD45.2+/CD4+/CD44lo/CD62Lhi gated cells (A) and the absolute numbers of donor naïve T cells (B) were determined. The data are mean ± SEM with n = 5 mice/group. *, p < 0.05.
Supplemental Figure 2

Supp. Figure 2

Increased number of thymocytes and peripheral T cells in middle-aged Bim+/- mice. Absolute number of CD4+CD8+Tg+ thymocytes in thymus (left), peripheral CD4 T cells (middle) and peripheral Tg+ naïve CD4 T cells (right) in spleen of indicated aged Bim+/- and Bim+/- AND Tg mice. The data are mean ± SEM with n = 5 mice/group. *, p < 0.05, **, p < 0.01.
Supplemental Figure 3

**Thymus**

**SP/LN**

Frequencies of CD45.1+ Bim+/+ and CD45.1- Bim+/− CD4+CD8-Tg+ thymocytes in the thymus (left 4 panels) and peripheral Tg+ naïve T cells in the spleen and peripheral LN (right 4 panels) from 3- and 9-mo old mixed BM chimeras were determined by flow cytometry.

Supp. Figure 3

Bim+/− naïve CD4 T cells dominates in vivo due to their increased survival. Frequencies of CD45.1+ Bim+/+ and CD45.1− Bim+/− CD4+CD8+ Tg+ thymocytes in the thymus (left 4 panels) and peripheral Tg+ naïve T cells in the spleen and peripheral LN (right 4 panels) from 3- and 9-mo old mixed BM chimeras were determined by flow cytometry.
Supp. Figure 4

Bim regulates the 1ge-related accumulation of functionally defective naïve T cells in the periphery. A, Bim+/+, Bim+/− and Bim−/− naïve AND Tg+ cells were isolated from mixed BM chimeras 7-mo after reconstitution, and then were stimulated, and then their proliferation stimulated with 0, 1, and 5 μM antigenic peptide-pulsed APC were determined by [3H] thymidine incorporation. B, At the indicated times after treatment with anti-CD4 Ab or Isotype-matched control Rat IgG, PBL were harvested from 6-mo old Bim+/+ and Bim+/− mixed BM chimeras and the frequencies of Tg+ naïve CD4 T cells was determined based on Vβ3, CD44 and CD4 expression. C, One month after Ab treatment, Bim+/+ and Bim+/− naïve AND Tg+ cells were purified from the 7-mo old mixed BM chimeras and then were stimulated with 0 or 5 μM antigenic peptide-pulsed APCs. The level of IL-2 in culture supernatants were accessed by the proliferative response of IL-2 responsive NK3 cell line. The values are the mean ± SEM with n = 5 mice/group; * p < 0.05, ** p < 0.01. The data are representatives from at least two independent experiments with similar results.
Supp. Figure 5

Antigen-driven proliferation defects in aged Bim^{+/−} naïve CD4 T cells \textit{in vivo}. Bim^{+/+} or Bim^{+/−} naïve AND T cells from 2.5- mo and 7-mo old mixed BM chimeras were individually transferred into ATxBM mice, and then those mice were immunized with NP-conjugated PCC antigen in Alum. Total number of donor Tg^{+} T cells was determined at indicated times after immunization.