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

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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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The online version of this article contains supplemental material.

Abbreviations used in this paper: ARF, alternative reading frame; ATxBM, adult thymectomized, lethally irradiated bone marrow-reconstituted mice; BM, bone marrow; C57BL/6; CBA, C57BL/6; C57BL/6; CBA, C57BL/6; MFI, mean fluorescence intensity; mo, not detected; NP, 4-hydroxy-3-nitrophenyl acetyl; PCC, pigeon cytochrome c; PNA, peanut agglutinin; SP, single-positive; Tg, transgenic; WT, wild-type.

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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-Pgp-1bWt+ Thy1+ and CD45.2 and biotinylated anti-CD45.1 Abs (eBioscience, San Diego, CA); Alexa Fluor 405-labeled anti-CD44 (Caltag Laboratories, Burlingame, CA); allophycocyanin-labeled anti-Thy1.1 (all purchased from BD Pharmingen, San Diego, CA); Fluorescein isothiocyanate (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-Thyl.2; allophycocyanin–allophycocyanin–anti-Thyl.1.1 (all purchased from BD Pharmingen, San Diego, CA); Alexa Fluoro 450–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–peanut agglutinin (PNA) was purchased from Invitrogen. 4-Hydroxy-3-nitrophenyl acetyl (NP)-conjugated allophycocyanin was prepared and stained with Pacific Blue-conjugated anti–IL-2 Ab (eBioscience, San Diego, 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 and Alexa Fluor 647 anti–CD44 Ab. 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−/+Vβ3+ CD44+ naive cells were sorted by FACSVantage 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 piggybacteriochrome c (PC) or BSS in alum.

Proliferation assays

Sorted naive AND TCR Tg T cells (2.5 × 10⁵/well) were stimulated with irradiated DCEK-ICAM/B7 cells (2.5 × 10⁵/well) and indicated concentration of PCC peptide in 96-well culture plates. After 60 h of coculture, T cells were pulsed with [³H]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 biosay 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-gp100 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 reverse transcription kits (Applied Biosystems, Foster City, CA). TaqMan probe for p19 alternative reading frame (p19ARF; Mm01257348, INKA4 (Mm0049444949), and INKB (Mm00483241) were obtained from Applied Biosystems. The primers and probes for GAPDH designed by Dr. S. Smiley and P. 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 (Ct) values for each reaction were determined and averaged using TaqMan SDS analysis software. The changes in gene expression were calculated by the comparative Ct method (fold changes = 2^-ΔΔCT).

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 (14). 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 lymphoplate 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).
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 prethymectomized 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<sup>−/−</sup> 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<sup>−/−</sup> AND Tg mice and determined the number and function of Bim<sup>−/−</sup> naive CD4 T cells from youth to middle age. Consistent with previous studies using non-TCR Tg mice (23), the number of CD4<sup>+</sup>CD8<sup>−</sup> single-positive thymocytes was significantly higher in young Bim<sup>−/−</sup> 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<sup>+</sup> naive CD4 T cells were also increased by Bim heterozygosity in both young and middle-aged mice. In both Bim<sup>+/−</sup> and Bim<sup>−/−</sup> mice, there was an age-dependent decrease in the numbers of total thymocytes and peripheral Tg<sup>+</sup> T cells, but the numbers in aged Bim<sup>−/−</sup> 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 AND Tg<sup>+</sup> T cells from Bim<sup>+/−</sup> and Bim<sup>−/−</sup> AND Tg mice of different ages were stimulated with cognate Ag, PCC peptide-pulsed APCs. Naive Tg<sup>+</sup> T cells from 2-mo-old Bim<sup>−/−</sup> 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−/− 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−/− T cells from 9 mo-old mice was less dramatic than that observed in T cells from 6 mo-old mice. Importantly, Bim−/− 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−/− 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−/− 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−/− 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−/− Tg+ 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−/− peripheral naive Tg+ CD4 T cells was slightly lower than those of WT cells in the mixed BM chimeras because of a smaller number of transferred Bim−/− BM cells (Fig. 3E, 4B). However, the rate of age-dependent progressive loss of the Bim−/− naive Tg+ T cell cohort was significantly lower than that of WT cells (Fig. 4B), suggesting increased persistence of Bim−/− 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−/− 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−/− 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−/− naive T cells from the 6.5-mo-old 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−/− 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−/− 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−/− naive T cells is largely responsible for their blunted proliferation.

To further evaluate the correlation between the development of functional defects and age of reduced Bim expression, we examined the TCR-mediated response of WT, Bim−/−, and Bim−/− naive CD4 T cells from 7-mo-old middle-aged BM chimeras. As shown in Fig. 4G, the middle-aged homozygous KO (Bim−/−) T cells produced less IL-2 than did Bim−/− 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+ T cells, and we detected new Tg+ CD4 T cells reconstituting the population during 3 wk (Supplemental Fig. 4B). Similar kinetics of reconstitution were seen for both WT and Bim−/− Tg+ T cells. One month after CD4 T cell depletion, we harvested the newly generated Bim−/− and Bim−/− naive CD4 T cells and stimulated them with cognate Ag. In control animals with no depletion, the Bim−/− cohort proliferated less, as we expected, but this defect was restored in the newly generated Bim−/− T cells in middle-aged BM chimeras after CD4 T cell depletion (Fig. 4H and Supplemental Fig. 4C).

**Bim−/− 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−/− naive CD4 T cells, we transferred Bim−/− or Bim−/− 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

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**FIGURE 3.** Age-related defects of peripheral naive CD4 T cells are exacerbated in heterozygous Bim−/− mice. A. Proliferation of naive CD4 T cells to Ag is shown. Cells purified from Bim−/− and Bim−/− AND Tg mice were stimulated with no peptide (−) or 5.0 μM (+) PCC peptide-pulsed APCs. B. IL-2 production by naive CD4 T cells is shown. Cells purified from Bim−/− and Bim−/− AND Tg mice were stimulated with increasing doses of PCC peptide (0.0 μM, left bars; 1.0 μM, middle bars; 5.0 μM, right bars), and IL-2 was measured in culture supernatants. Results are presented as the mean ± SEM of triplicate cultures. *p < 0.05; **p < 0.01.
RESULTS are presented as the mean triplicate assay. The proliferative response (C) and IL-2 production (D) of Bim"++" and Bim"+-" 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"++" and Bim"+-" naive AND Tg cells were isolated from BM chimeras 6 mo after reconstitution. IL-2 production in CD4"+Vβ"3" populations in response to cognate Ag was determined by intracellular cytokine staining. F, Naïve AND T cells as described in E were stimulated without or with 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 CD4 T cells from the middle-aged chimeras had divided less at day 4 and much less at day 6, confirming a defect in proliferation (Fig. 5A). Although the rate of division of Bim"+-" T cells was slower, the total number of donor Bim"+-" T cells was comparable to that of WT T cells by day 12 after immunization (Supplemental Fig. 5), probably because Bim"+-" T cells underwent less apoptosis than did Bim"++" T cells after Ag clearance (28).

To evaluate cognate help, we analyzed the expansion and differentiation of NP-specific B cells in the recipients of naïve CD4 T cells isolated from 7-mo-old mixed BM chimeras. Fifteen days after immunization, we found a 3-fold increase of NP-specific B cells in Ag-primed mice compared with adjuvant control mice with WT T cells, whereas the increase was <2-fold in mice that received Bim"+-" T cells (Fig. 5B). We also quantified PNA"+CD38"lo germinal center (GC) phenotype cells within the NP-specific B cell population. In recipients of WT naïve CD4 T cells, a large population of NP B cells differentiated into GC B cells (71.6%), whereas in the recipients of middle-aged Bim"+-" T cells, the proportion was 2-fold less (35%), and the number of GC phenotype NP B cells was reduced significantly (Fig. 5C, 5D). Because the GC is the site of Ig isotype class switching, we analyzed the expression of IgM and IgG1 on GC B cells (Fig. 5C, lower panels). The ratio of switched IgG1"+" B cells to nonswitched (IgM") in the recipients of Bim"+-" T cells was 3-fold lower than those with WT T cells (WT, 18.3; Bim"+-", 5.7%). Additionally, recipients of Bim"+-" CD4 T cells had fewer NP"+PNA"+ B cell-expressing IgG1 (Fig. 5C, 5D), indicating a loss of help for T cell-driven B cell expansion. Reduced Bim expression in T cells did not in and of itself cause impaired GC formation or class switching since NP-specific GC B cells and IgG1"+" B cells were equivalent in recipients of Bim"+-" and Bim"+-" naïve T cells derived from 2.5-mo-old young mixed BM chimeras, confirming that the defect in helper activity caused by Bim insufficiency did not develop until middle age.

We also measured Ag-specific Ab production by determining the titer of NP-specific IgG1 in the sera of the mice that received donor Bim"+-" and Bim"++" T cells from the 2.5- and 7-mo-old chimeras. In recipients of the young Bim"+-" and Bim"++" naive CD4 T cells, there were comparable NP-specific IgG1 titers, confirming that the naive CD4 T cell expressing lower Bim could nonetheless provide normal cognate B cell helper activity (Fig. 5E). In contrast, when the donor cells were recovered after 7 mo, the recipients of Bim"+-" T cells had significantly lower titers of anti-NP-specific IgG1 than did the recipients of WT T cells at all time points. These results indicate that by middle age the longer lived Bim"++, T cells
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 INK4a in the recipients of young and middle-aged Bim+/+ or Bim−/− naive AND T cells are shown. Serum was harvested at indicated time from the recipient mice that were described in D. The Ab titers in the recipients of 2.5-mo-old Bim+/+ versus Bim−/− donor cells did not differ significantly. Data from two independent experiments are combined. *p < 0.05; **p < 0.01.

**FIGURE 5.** Bim reduction accelerated development of age-related effects in helper cell-dependent Ab production. A, One million CFSE-labeled Bim+/+ or Bim−/− naive AND T cells from 7-mo-old mixed chimeras were transferred into ATxBM mice, and recipients were immunized with NP-conjugated PCC Ag in alum. The in vivo Ag-driven proliferation of middle-aged donor naive CD4 T cells was evaluated by CFSE dilution at indicated times after immunization. B and C, ATxBM recipients of Bim+/+ or Bim−/− naive AND T cells from 7-mo-old chimeras were immunized with PBS or NP-conjugated PCC Ag in alum, and then spleen cells were analyzed 15 d after immunization. The proportions of NP-binding B cells (B), CD38 and PNA expression by B220+NPs cells (C, upper panels), and IgM or IgG1 expression by B220+NPs+B220− cells (C, lower panels) are shown. C, Number of NP-specific GC B cells is shown. Bim+/+ or Bim−/− naive AND T cells were isolated from 2.5- or 7-mo-old chimeras and cells were transferred into ATxBM mice. They were immunized as described in A. At day 15 after immunization, absolute numbers of NP−CD38−PNA− B cells (left panel) and NP−CD38−PNA−IgG1− B cells (right panel) were determined. The values are the mean ± SEM with n = 4–5 mice/group. D, 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).
Expression of markers of cellular aging is upregulated in long-lived naive CD4 T cells. A, Expressions of INK4a, INK4b, and p19ARF in 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 (top). Newly generated naive AND T cells were isolated from BM chimeras reconstituted with BM cells from young or aged AND Tg mice described above (bottom). mRNA expression for the INK family members determined by real-time RT-PCR. B, Levels of INK family members expressed by Bim+/+ and Bim−/− naive AND Tg+ T cells from 2.5- and 10-mo-old mixed BM chimeras are shown. Relative mRNA expression of indicated genes was analyzed as described in A. All data are normalized by each GAPDH expression and are expressed as the fold difference (mean ± SEM) compared with the average value of the young WT naive Tg+ T cells (n = 3–4). *p < 0.05.

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 (Fig. 6B). 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 lifespan 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 naive 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 naive 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 naive 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 naive 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(WT) naive 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/ionomycin cannot restore the defective IL-2 production in aged T cells (14), and aged naive 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 naive 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 naive 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 naive CD4 T cells derived from aged BM expressed lower levels than did long-lived cells. INK family expression in young Bim(WT) and WT cells was equivalent, but INK4a and p19ARF increased more quickly with age in the Bim(CD4−) cells. Thus, their expression is also correlated with cellular age in naive CD4 T cells.

There are some key differences between classically defined replicative senescence and the loss of function in naive T cells with aging. First, whereas replicative senescence is caused by extensive proliferation (32), naive 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 naive CD4 T cells are quiescent and nondenviding, 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 naive 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 naive CD4 T cells from aged mice (14, 45, 46) and the long-lived cells from middle aged Bim(WT) T cells (Fig. 4). Thus, we suggest that the erosion in function that occurs in the naive 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 naive 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 naive 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 naive 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 10^6) 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+CD44loCD62Lhi 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

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. *, \(p < 0.05\), **, \(p < 0.01\).
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.
Supplemental Figure 4

Supp. Figure 4

Bim regulates the 1ge-related accumulation of functionally defective naïve T cells in the periphery. A, Bim<sup>+/+</sup>, Bim<sup>+</sup><sub>−</sub> and Bim<sup>−/−</sup> naïve AND Tg<sup>+</sup> 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<sup>+/+</sup> and Bim<sup>−/−</sup> mixed BM chimeras and the frequencies of Tg<sup>+</sup> naïve CD4 T cells was determined based on Vβ3, CD44 and CD4 expression. C, One month after Ab treatment, Bim<sup>+/+</sup> and Bim<sup>+</sup><sub>−</sub> naïve AND Tg<sup>+</sup> 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.
Antigen-driven proliferation defects in aged Bim\textsuperscript{+-} naïve CD4 T cells \textit{in vivo}. Bim\textsuperscript{++} or Bim\textsuperscript{+-} 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\textsuperscript{+} T cells was determined at indicated times after immunization.