Proliferation and Regulate Homeostatic Naive T Cell
Dendritic Cells

Expression of IL-27 in CD8+ Dendritic Cells and Regulate Homeostatic Naive T Cell Proliferation

Jeong-su Do, Anabelle Visperas, Keunhee Oh, Stephen A. Stohlman and Booki Min

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Memory CD4 T Cells Induce Selective Expression of IL-27 in CD8+ Dendritic Cells and Regulate Homeostatic Naive T Cell Proliferation

Jeong-su Do,* Anabelle Visperas,*† Keunhee Oh,*† Stephen A. Stohlman,* and Booki Min*‡

Naive T cells undergo robust proliferation in lymphopenic conditions, whereas they remain quiescent in steady-state conditions. However, a mechanism by which naive T cells are kept from proliferating under steady-state conditions remains unclear. In this study, we report that memory CD4 T cells are able to limit naive T cell proliferation within lymphopenic hosts by modulating stimulatory functions of dendritic cells (DC). The inhibition was mediated by IL-27, which was primarily expressed in CD8+ DC subsets as the result of memory CD4 T cell–DC interaction. IL-27 appeared to be the major mediator of inhibition, as naive T cells deficient in IL-27R were resistant to memory CD4 T cell–mediated inhibition. Finally, IL-27–mediated regulation of T cell proliferation was also observed in steady-state conditions as well as during Ag-mediated immune responses. We propose a new model for maintaining peripheral T cell homeostasis via memory CD4 T cells and CD8+ DC-derived IL-27 in vivo. The Journal of Immunology, 2012, 188: 230–237.

As a result, T cells displaying memory phenotypes are generated, although only a few millions of these cells are typically found in the lymphoid tissues of these recipients (11–13). Because transferred naive T cells either differentiate into memory phenotype cells or die, and there is no endogenous source of naive T cells in these hosts, the lymphopenic status remains unchanged except for a relatively small number of memory phenotype T cells derived from the initial transfer. Importantly, those memory phenotype T cells are fully capable of inhibiting the proliferation of naive T cells that are newly transferred into the recipients (12, 14). How naive T cells are kept from proliferating in memory T cell–enriched lymphopenic conditions has not been previously explored. Thus, understanding mechanism(s) underlying the proliferation may provide fundamental insight into the regulation of homeostatic T cell proliferation.

One key player involved in T cell activation/proliferation is APC, particularly dendritic cells (DC). In addition to inducing T cell immunity postinfection or immunization, DC are also critical for naive CD4 T cells to undergo proliferation in lymphopenic hosts (15). DC also deliver tolerogenic signals (16); it was recently demonstrated that DC acquire IL-27–dependent regulatory functions, inducing IL-10–producing T cell tolerance and suppressing autoimmune neuroinflammation (16). Consistent with this, IL-27R−/− or IL-27−/− mice were highly susceptible to the disease and generated more IL-17+ encephalitogenic T cells (17, 18). IL-27 also suppresses CD28-mediated IL-2 production and T cell proliferation via suppressor of cytokine signaling 3 (19–21).

In this study, we examined the hypothesis that memory phenotype CD4 T cells (which will be referred to as memory T cells hereafter) inhibit naive T cell proliferation by altering stimulatory functions of APC. Memory CD4 T cells fully inhibited the proliferation of both naive CD4 and CD8 T cells in lymphopenic hosts. This inhibition was found only when both naive and memory T cells interact with the same APC (i.e., the inhibition was abolished when memory CD4–APC interaction was absent even under memory cell–enriched conditions). The expression of IL-27 was found elevated when naive T cell proliferation was inhibited. Naive T cells deficient in IL-27R underwent robust proliferation.
regardless of the presence of memory T cells in vivo. CD8+ DC were the dominant population that expressed high levels of IL-27 following CD4 T cell–DC interaction. IFN-γ was necessary to induce IL-27 expression in CD8+ DC. Therefore, IL-27 expressed by DC directly controls naive T cell proliferation, and the memory CD4 T cell interaction with the DC controls the IL-27 expression. Our results highlight a novel feedback mechanism involving memory CD4 T cells and DC derived IL-27, from which homeostatic regulation of naive T cells is kept under control.

Materials and Methods

Mouse

Ly5.1 C57BL/6, Thy1.1 C57BL/6, C57BL/6 TCRβ+−, C57BL/6 MHC class II (MHC II) + (H2Kd−/−), C57BL/6 Rag2+−, C57BL/6 OT-II TCR Tg, C57BL/6 IFN-γR−/−, C57BL/6 IFN-γ−/−, and C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B10.A Rag2−/−, and Ly5.1 B10.A mice were kindly provided from Dr. William Paul (National Institutes of Health). C57BL/6 IL-27R−/− mice were provided from Amgen (Thousand Oaks, CA). All of the mice were maintained under specific pathogen-free facility located in the Lerner Research Institute. All animal experiments were performed in accordance with approved protocols for the Cleveland Clinic Foundation Institutional Animal Care and Use Committee.

Cell isolation and adoptive transfer

Donor naive T cells were isolated from lymph nodes. In brief, lymph node cells were incubated with FITC-conjugated anti-MHC II (M5/114), anti-FcγR (2.4G2), anti-NK1.1 (PK136), and anti-B220 (RA3-6B2) Abs. Labeled cells were subsequently incubated with anti-FITC microbeads (Miltenyi Biotec, Auburn, CA), and then T cells were purified through magnetic field isolation. Cells were then labeled with PE-anti-CD44 (IM7) and allopheocyanin–anti-CD4 (RM4-5) or allopheocyanin–anti-CD8 (53-6.7). CD44low naive T cells were sorted using a FACSAria high-speed cell sorter (BD Biosciences, Franklin Lakes, NJ). Purity is typically >99%. As the primary transfer, Rag−/− or TCRβ−/− mice were transferred with 3 × 106 purified CD4 T cells. Four weeks later, the mice received the secondary naive T cells. A total of 1 × 106 naive T cells were i.v. transferred into recipients. In some experiments examining cell proliferation, T cells were labeled with CFSE (Molecular Probes, Carlsbad, CA). The recipients were sacrificed 7 d posttransfer, and proliferation (as well as total cell recovery) was determined by FACs analysis using an FACSCalibur or an FACS LS II (BD Biosciences) and FlowJo software (Tree Star, Ashland, OR). The following Abs were used: anti-CD4 (RM4-5), anti-Ly5.1 (A20), anti-Thy-1.1 (BHI51), anti-CD8 (53-6.7), and anti-I–Aγ (AF6-120.1), and anti-I–Eα (AF6-88.5), and anti-I–Eβ (14-4-4S). All Abs were purchased from eBioscience (San Diego, CA) or BD Pharmingen (San Diego, CA). In some experiments examining T cell proliferation in vivo, the recipients were injected with 1 mg BrdU 1 d prior to sacrifice. BrdU incorporation was determined using a BrdU staining kit (BD Pharmingen) according to the manufacturer’s manual. In some experiments, recipients were s.c. immunized with OVA protein (Sigma-Aldrich) plus LPS (Sigma-Aldrich).

Bone marrow chimeras

Lethally irradiated mice were transferred with 15–20 × 106 bone marrow (BM) cells isolated from the donor mice as indicated in the text. The BM recipients were i.p. injected with gentamicin at days 0 and 2 posttransfer. Six weeks later, the recipients were bled, and reconstitution was confirmed prior to the transfer experiments. In the experiments examining CD4-mediated CD4 T cell proliferation described in Fig. 2, the BM chimeric recipients were reinfused with anti-NK1.1 Ab (250 µg/mouse, −1 and 3 d) prior to the secondary donor CD4 T cell transfer. In some experiments, a 1:1 mixture of Ly5.1 WT and Ly5.2 IL-27R−/− BM cells was injected into lethally irradiated recipients. Reconstitution of each T cell type was monitored every 2 wk postreconstitution.

Real-time quantitative PCR

CD11c+ splenic DC were isolated from wild-type (WT), Rag−/−, and Rag−/− mice that received CD4 T cells 4 wk earlier. DC were further sorted into different subsets based on the expression of CD8. Total RNA was extracted using an RNeasy column (Qiagen, Valencia, CA). cDNA was subsequently obtained using a SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). Real-time PCR was performed using the Ebi3- and p28-specific primers and probe sets (Applied Biosystems, Foster City, CA) and ABI 7500 PCR machine (Applied Biosystems). In some experiments, BM-derived DC were generated and cocultured with OT-II CD4 T cells with or without 1 µM OVA peptide for 48 h. CD8+ and CD8+ CD11c+ DC from the culture were further sorted by FACS and subsequently analyzed for the expression of IL-27 subunits. All gene expression results are expressed as arbitrary units relative to expression of the endogenous control GAPDH. Relative expression of each gene is quantified using the formula 2−ΔΔCT.

Data analysis

Statistical significance was determined by the Student t test using Prism 4 software (GraphPad, La Jolla, CA). A p value <0.05 was considered to indicate a significant difference.

Results

Experimental model

T cell proliferation induced by a homeostatic mechanism is determined by the lymphopenic status of hosts; naive T cells remain quiescent in lymphocyte-sufficient but rapidly proliferate in lymphocyte-deficient hosts. We and others previously reported that memory CD4 T cells play a key role in limiting naive CD4 T cell proliferation (12, 14). To investigate the underlying mechanism, we employed a double transfer strategy as previously reported (Supplemental Fig. 1) (12). CD4 T cells (1′ transfer) were introduced into lymphopenic mice (Rag−/− or TCRβ−/−), allowing them to expand, differentiate into memory cells, and populate the peripheral lymphoid tissues of the recipients. Four weeks later, the recipients received a new cohort of CFSE-labeled Thy1.1 (or Ly5.1 in some experiments) CD44low naive T cells (2′ transfer), and the CFSE dilution was determined 7 d after transfer. Of note, ~100% of the 1′ CD4 T cells acquired a memory phenotype, although their cellularity within the secondary lymphoid tissues was ~2 × 106 at the time of the 2′ transfer, indicating a severe lymphopenic status of the recipients (12, 22). As previously reported (12), the pre-existing 1′ memory CD4 T cells fully inhibited the proliferation of 2′ naive CD4 T cells (Fig. 1A). Interestingly, memory CD4 T cells inhibited naive CD8 T cell proliferation as well (Fig. 1A). The inhibition was not found without the 1′ transfer (Fig. 1B). Although it was previously proposed that memory T cells with a given specificity may prevent responses of naive T cells of the same or related specificity (23), CD4 and CD8 T cells are not likely to share specificity; thus, an alternative mechanism may operate.

FIGURE 1. Pre-existing memory phenotype CD4 T cells inhibit naive T cell proliferation. A. Groups of Rag−/− mice were transferred with 3 × 106 Thy1.2 CD4 T cells (1′ CD4). Subsequently, the recipients were transferred with CFSE-labeled FACS-sorted Thy1.1 naive 2′ T cells (1 × 106, containing both CD4 and CD8) 4 wk post-1′ CD4 transfer. Histograms shown are CFSE profiles of Thy1.1-gated T cells (2′ CD4 or 2′ CD8) examined 7 d posttransfer. B. CFSE-labeled Thy1.1 naive T cells were transferred into Rag−/− mice. CFSE profiles were determined 7 d posttransfer. The experiments were repeated three times, and similar results were observed. The average ± SD of the proportion of T cells that fully diluted CFSE is indicated. Similar results were found in the spleen (not shown). LN, peripheral lymph node; mLN, mesenteric lymph node.
Memory CD4 T cell-mediated inhibition of naive CD8 T cell proliferation operates through APC

Interaction of T cells expressing high-affinity Ag receptors with APC can downregulate peptide–MHC complexes on the APC, preventing further activation of newly recruited naive T cells (24). We thus hypothesized that memory CD4 T cells may down-modulate stimulatory functions of APC and that this interaction between memory CD4 T cells and APC may be essential to restrain naive cell proliferation. The hypothesis also predicts that APC that do not interact with memory CD4 T cells may preserve stimulatory functions even in the presence of them. To test this hypothesis, BM chimeras in which interactions between APC and memory CD4 T cells are restricted were generated. Irradiated Rag\(^{-/-}\) mice were reconstituted with BM cells from TCR\(^{\beta/-}\) (Fig. 2A) or with a 1:1 mixture of BM cells from TCR\(^{\beta/-}\) and TCR\(^{\beta/-}\) MHC II\(^{-/-}\) mice (Fig. 2B). The recipients contained T cell-deficient but had APC expressing both MHC class I (MHC I) and II (Fig. 2A) or two APC populations, one expressing both MHC I and II and one expressing MHC I alone (Fig. 2B). MHC expression on CD11c\(^+\) splenic DC from these chimeras confirmed the appropriate reconstitution (Fig. 2A, 2B). The reconstituted mice received 1’ CD4 T cells and CFSE-labeled naive 2’ CD8 T cells 4 wk later. Consistent with results shown in Fig. 1, CD8 T cells failed to proliferate in mice in which APC expressed both MHC I and II (Fig. 2A, right panel). By contrast, a substantial proliferation was found in recipients in which \(\sim 50\%\) of the APC were MHC II\(^{-/-}\) (Fig. 2B, right panel). This proliferation was not due to different numbers of 1’ memory CD4 T cells generated in these recipients because the total numbers of CD4 T cells derived from the 1’ transfer were similar (Fig. 2F). CD8 T cells underwent robust proliferation in both types of BM chimeras without 1’ CD4 transfer (Fig. 2A, 2B, left panels) or in control TCR\(^{\beta/-}\) recipients (Fig. 2C). Therefore, memory CD4 T cell interaction with MHC II\(^{-/-}\) APC may limit the proliferation of naive CD8 T cells that interact with the same APC.

Memory CD4 T cell-mediated inhibition of naive CD8 T cell proliferation operates through APC

To examine if memory CD4 T cells similarly regulate naive CD8 proliferation, we used CD4 T cells restricted to different MHC II haplotype molecules (illustrated in Supplemental Fig. 2). To establish recipients in which APC express different MHC II restriction elements, B6 \(\times\) B10.A F1 Rag\(^{-/-}\) mice were reconstituted with B6 \(\times\) B10.A F1 (I-A\(^{\text{bkx}}\)) Rag\(^{-/-}\) BM (Fig. 2D) or a 1:1 mixture of B6 (I-A\(^{\text{b}}\)) Rag\(^{-/-}\) and B10.A (I-A\(^{\text{b}}/\text{I-E}^{\text{k}}\)) Rag\(^{-/-}\) BM cells (Fig. 2E). APC from the former recipients are expected to express both I-A\(^{\text{b}}\) and I-A\(^{\text{b}}/\text{I-E}^{\text{k}}\) (Fig. 2D), whereas APC from the latter recipients are expected to express either I-A\(^{\text{b}}\) or I-A\(^{\text{b}}/\text{I-E}^{\text{k}}\) (Fig. 2E). Appropriate reconstitution was confirmed by examining MHC II expression in splenic DC at 6 wk after reconstitution (Fig. 2D, 2E).

Because of alloreactivity, CD4 T cells that are restricted to one MHC II but tolerant to another MHC II element were needed. Because developing CD4 T cells are restricted to selecting MHC II during thymic development (25), B10.A BM cells transferred into...
lethally irradiated B6 Rag-/- mice are expected to develop into CD4 T cells that are restricted to I-A<sup>b</sup> but tolerant to I-A<sup>q</sup>/I-E<sup>b</sup> (Supplemental Fig. 3A). Likewise, B6 BM cells transferred into lethally irradiated B10.A Rag-/- mice are expected to develop into CD4 T cells that are restricted to I-A<sup>a</sup>/I-E<sup>b</sup> but tolerant to I-A<sup>q</sup> (Supplemental Fig. 3A). To examine if T cells obtained above behave as expected a proof-of-principle experiment was performed. Sorted naive CD4 T cells from B10.A Rag-/- recipients reconstituted with B6 BM cells (I-A<sup>a</sup>/I-E<sup>b</sup>-restricted/I-A<sup>a</sup> tolerant) were transferred into B10.A Rag-/- or B6 Rag-/- recipients, and the proliferation was assessed. As expected, those CD4 T cells fully diluted the CFSE content only within I-A<sup>a</sup>-expressing B10.A Rag-/- but not within I-A<sup>b</sup>-expressing B6 Rag-/- recipients (Supplemental Fig. 3B, filled arrow), indicating an MHC II-restricted T cell proliferation. Interestingly, IL-7-dependent homeostatic proliferation (4, 8) (i.e., one to two cell divisions) was still observed in B6 Rag-/- recipients (Supplemental Fig. 3B, open arrow), suggesting that the cytokine-dependent proliferation is not an MHC-restricted response, although MHC II molecules are still necessary (4, 8).

CD4 T cells (1') restricted to I-A<sup>b</sup> MHC II elements were isolated and then transferred into reconstituted recipients described in Fig. 2D and 2E. I-A<sup>a</sup>/I-E<sup>b</sup>-restricted (and CFSE-labeled) 2' naive CD4 T cells were transferred 4 wk after the 1' CD4 transfer. Consistent with the results seen in CD8 T cells, the pre-existing I-A<sup>a</sup>-restricted memory CD4 T cells efficiently inhibited 2' CD4 T cell proliferation when APC expressed both MHC II haplotype molecules (Fig. 2D, right panel). By contrast, the same 2' naive CD4 T cells underwent robust proliferation when transferred into recipients in which I-A<sup>a</sup>/I-E<sup>b</sup>-expressing APC (Fig. 2E, open arrow) do not express I-A<sup>b</sup> and therefore do not interact with memory 1' CD4 T cells (Fig. 2E, right panel). Notably, the total numbers of memory 1' CD4 T cells in both recipients were similar, indicating that the different proliferation of 2' naive T cells is not due to a discrepancy in the memory CD4 T cell numbers present in these recipients (Fig. 2G). Robust proliferation of 2' CD4 T cells was equally observed in both types of recipients without 1' CD4 T cell transfer (Fig. 2D, 2E, left panel). Therefore, these results strongly suggest that APC play a central role in controlling naive T cell proliferation and that memory CD4 T cells directly control the stimulatory function of the APC via TCR-MHC II interactions.

**Continuous presence of memory CD4 T cells is necessary to mediate the inhibition**

If stimulatory functions of APC are downregulated by memory CD4 T cells, those APC may remain nonstimulatory, or alternatively, continuous presence of memory CD4 T cells may be required to maintain the functions. To directly test this possibility, pre-existing memory CD4 T cells expressing Thy1.2 were depleted using anti-Thy1.2 Ab when naive T cells expressing Thy1.1 were transferred. As shown in Fig. 3, the proliferation of both Thy1.1 naive CD4 and CD8 T cell proliferation was significantly restored upon memory cell depletion. A partial restoration of Thy1.1 T cell proliferation could be due to a homeostatic competition by residual nondepleted memory T cells that are also likely to undergo proliferation under this condition (26). Our results suggest that APC-mediated control of naive T cell proliferation is a reversible process that is directly dependent on the presence of memory CD4 T cells.

**IL-27 mediates memory CD4 T cell-induced inhibition of naive T cell proliferation**

To examine mechanism(s) underlying APC-mediated control of naive T cell proliferation, we first compared surface expression of molecules involved in T cell activation/inhibition. No significant differences in the expression of MHC as well as costimulatory molecules were found in CD11c<sup>+</sup> DC isolated from WT and lymphopenic TCR<sup>B<sup>/-</sup></sup>- (and Rag<sup>-/-</sup>, data not shown) mice. IL-27 has recently been shown to be induced by DC that deliver tolerogenic signals to T cells (16) and to suppress T cell IL-2 production (19). To directly test if IL-27 is involved, we used naive CD4 T cells deficient in IL-27R (20). CD4 T cells were transferred into TCR<sup>B<sup>/-</sup></sup>- mice. Four weeks later, CFSE-labeled CD4<sup>+</sup> naive IL-27R<sup>-/-</sup> or WT CD4 T cells were transferred into the recipients. To our surprise, IL-27R<sup>-/-</sup> CD4 (Fig. 4A) and CD8 (data not shown) T cells were refractory to memory T cell-induced inhibition, whereas WT CD4 T cell proliferation was fully suppressed. Of note, the proliferation of WT and IL-27R<sup>-/-</sup> T cells was similar in lymphopenic conditions without the 1' CD4 transfer (data not shown), suggesting that IL-27R<sup>-/-</sup> T cells are...
not more prone to proliferation compared with WT cells in the absence of memory CD4 T cells. Therefore, IL-27 expressed in the presence of memory CD4 T cells appears to signal the IL-27R on naive CD4 T cells and directly prevents the proliferation.

Because activated APC are the major source of IL-27 (27), and DC are the primary APC inducing proliferation of both naive and memory CD4 T cells in lymphopenic settings (15), we next examined if DC mediate the inhibition by releasing IL-27. CD11c+ DC were isolated from WT and Rag \(^{-/-}\) mice and examined for the expression of IL-27 subunits Ebi3 and p28. Indeed, the expression of both subunits was significantly diminished in DC isolated from lymphopenic Rag \(^{-/-}\) mice compared with those DC isolated from wild-type mice (Fig. 4B). Importantly, IL-27 expression in DC dramatically increased when Rag \(^{-/-}\) mice received CD4 T cells (Fig. 4B). Likewise, IL-27 expression was lower in DC isolated from TCR \(^{b}\) b-transferred with CD4 T cells. IL-27 expression in CD11c+ splenic DC was expressed by a subset of DC, we isolated different CD11c+ DC subsets from WT mice based on the expression of CD8 and measured IL-27 expression. We found that IL-27 subunits were expressed only in CD8+ DC subsets (Fig. 6A). Selective expression of IL-27 in CD8+ DC was further confirmed in DC–OT-II coculture experiments. The addition of Ag in the culture significantly increased IL-27 expression only in CD8+ DC subsets, whereas CD8–CD11c+ DC expressed little IL-27 (Fig. 6A). Selective expression of IL-27 in CD8+ DC was further confirmed in DC–OT-II coculture experiments. The addition of Ag in the culture significantly increased IL-27 expression only in CD8+ DC subsets, whereas CD8–CD11c+ DC expressed little IL-27. Indeed, the expression was also restored following CD4 T cell transfer (data not shown). IL-27 expression increased as early as 7 d after CD4 transfer, and it continued to increase up to 4 wk posttransfer (Fig. 5A). IL-27 expression was mostly found in DC, and B cells expressed little IL-27, suggesting that DC are the primary source of IL-27 (Fig. 5B). Therefore, the lack of CD4 T cells appears to be responsible for the defects in IL-27 expression.

IL-27 is highly expressed in CD8+ DC

Functional specialization of DC subsets particularly in preventing self-reactive responses has been described (28). To examine if IL-27 seen in WT mice or lymphopenic mice that receive CD4 T cells is expressed by a subset of DC, we isolated different CD11c+ DC subsets from WT mice based on the expression of CD8 and measured IL-27 expression. We found that IL-27 subunits were primarily expressed in CD8+ DC subsets, whereas CD8–CD11c+ DC expressed little IL-27 (Fig. 6A). Selective expression of IL-27 in CD8+ DC was further confirmed in DC–OT-II coculture experiments. The addition of Ag in the culture significantly increased IL-27 expression only in CD8+ DC subsets, whereas CD8–CD11c+ DC expressed little IL-27. Indeed, the expression was also restored following CD4 T cell transfer (data not shown). IL-27 expression increased as early as 7 d after CD4 transfer, and it continued to increase up to 4 wk posttransfer (Fig. 5A). IL-27 expression was mostly found in DC, and B cells expressed little IL-27, suggesting that DC are the primary source of IL-27 (Fig. 5B). Therefore, the lack of CD4 T cells appears to be responsible for the defects in IL-27 expression.

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Homeostatic role of IL-27 in steady-state conditions

Although IL-27 controls T cell proliferation under lymphopenic settings, whether it plays a similar role in a nonlymphopenic setting was next examined. Mice deficient in IL-27R were reported healthy and fertile, and no significant differences in gross or in body weights between wild-type and deficient animals were found (20). Moreover, development and differentiation of lymphocytes in the lymphoid tissues were found to be normal in these mice (20). However, we noticed that in vivo proliferation of CD44\textsuperscript{high} memory phenotype T cells was substantially higher in IL-27R\textsuperscript{-/-} mice than in WT mice (Fig. 8A). The importance of IL-27R\textsuperscript{-/-} T cells in T cell expansion was better demonstrated in Rag \(^{-/-}\) mice that are reconstituted with a 1:1 mixture of Ly5.1 WT and Ly5.2 IL-27R\textsuperscript{-/-} BM cells. As shown in Fig. 7B, IL-27R\textsuperscript{-/-} BM-derived T cells significantly outgrew WT BM-derived T cells over time; ~80% of peripheral T cells were of IL-27R\textsuperscript{-/-} BM origin. In contrast, equal reconstitution of Ly5.1 WT and Ly5.2 WT T cells was found in control mice (Fig. 8B). Of note, comparable repopulation of WT and IL-27R\textsuperscript{-/-} T cells at an early time postreconstitution strongly suggests that the outgrowth of IL-27R\textsuperscript{-/-} T cells is not due to a competitive advantage during thymic development (Fig. 8B). Both IL-27R\textsuperscript{-/-} CD4 (Fig. 8C) and CD8 T cells (data not shown) from the reconstituted mice displayed a greater proliferation activity compared with WT T cells. On the contrary, equal proliferation was found in control mice (data not shown). Con-
sistent with these results, CD4\textsuperscript{high} memory phenotype T cells generated from IL-27R\textsuperscript{−/−} BM cells were substantially abundant than those generated from WT BM cells (Supplemental Fig. 4). Therefore, IL-27 may regulate the generation as well as the maintenance of memory phenotype T cells in steady-state conditions.

Lastly, whether IL-27 plays a role in the generation of Ag-specific effector CD4 T cells was examined. WT B6 mice were transferred with WT or IL-27R\textsuperscript{−/−} OT-II CD4 T cells and subsequently immunized with OVA. Expansion of OT-II T cells was then monitored. As shown in Fig. 8D, the expansion of IL-27R\textsuperscript{−/−} OT-II T cells was significantly greater than that of WT OT-II T cells, further suggesting a key regulatory role of IL-27 during Ag-mediated T cell responses.

**Discussion**

Lymphocyte levels in the periphery determine proliferative behaviors of naive T cells; in lymphopenic conditions, naive T cells proliferate, whereas in steady-state conditions, they remain quiescent. Efforts have been made to understand underlying mechanisms that induce T cell proliferation in lymphopenic hosts (4). However, how T cells are prevented from proliferating under steady-state conditions has not been formally explored. We report in this study that naive T cell proliferation is primarily determined by stimulatory functions of APC particularly of DC (23) and that memory CD4 T cells directly modulate the conditions of the DC. We identified that IL-27 produced by CD8\textsuperscript{+} DC subsets through interaction with memory CD4 T cells plays a central role in controlling naive T cell proliferation by directly acting on naive T cells. Furthermore, IL-27–mediated regulation of T cell proliferation was also found in resting conditions as well as Ag-mediated immune responses.

A simplistic view of T cell homeostasis would be a soluble factor-mediated competition between lymphocytes. In steady-state conditions, factors available to T cells may be sufficient to maintain survival but not enough to induce proliferation, whereas in lymphopenic conditions, increased availability of these factors may deliver signals sufficient to induce proliferation. IL-7 has been well characterized to explain this model (34–37). Indeed, IL-7 levels in the circulation inversely correlate with T cell levels (37, 38). However, a cytokine-independent (TCR-MHC–dependent) proliferation found in lymphopenic hosts (4, 8), which is the major response of interest in this study, is not supported by this model. Moreover, this is the dominant response observed within severe lymphopenic settings and is believed to result in immunopathology that often develops in such conditions.

Our results provide key mechanisms that explain a fundamental basis of T cell homeostasis. First, memory phenotype CD4 T cells are the regulators of peripheral T cell homeostasis. Rag\textsuperscript{−/−} mice populated with memory cells efficiently prevent newly transferred naive T cells from proliferating (12, 23). Memory Marilyn TCR-transgenic CD4 T cells transferred into Rag\textsuperscript{−/−} recipients suppress naive Marilyn CD4 T cell proliferation (14). Eliminating these memory T cells allowed naive T cells to proliferate. Therefore, we would argue that the lack of memory T cells is the primary driving force of naive T cell proliferation. Once memory cells occupy the peripheral lymphoid tissues, it becomes a homeostatically stable environment regardless of naive T cells, as demonstrated in our study.

Second, memory CD4 T cells regulate naive cell responses through APC. This type of regulation was previously reported during immune responses. CD4 T cells engage and condition DC...
through a CD40L--CD40 interaction, and these DC can promote an optimal CD8 differentiation into cytotoxic effector cells (39). Likewise, orally immunized memory T cells use IL-4 and IL-10 to educate Ag-presenting DC, which then induce naïve T cells to produce the same cytokines as those produced by memory T cells (40). Our results indicate that in lymphopenic (and noninflammatory) and even in steady-state conditions, a similar type of regulatory mechanism may operate. Memory CD4 T cells interact with APC, particularly CD8+ DC, alter their stimulatory functions, and in turn prevent naïve T cell proliferation. CD40L--CD40 interaction was not involved in this process as memory CD4 T cells within CD40-deficient Rag2−/− mice were fully capable of inhibiting naïve T cell proliferation (data not shown).

Third, IL-27 expressed by CD8+ DC subsets directly inhibits naïve T cell proliferation. IL-27 plays both pro- and anti-inflammatory roles in both innate and adaptive immunity (27). IL-27 production by APC can be induced by inflammatory-mediated signals associated with pathogens (41, 42). The activation of the NF-κB pathway might induce high levels of IL-27 production, which may synergize with IL-12 to promote Th1 immunity (20, 42). Under noninflammatory lymphopenic conditions described in this study, IL-27 expression in DC appears to be mainly controlled by IFN-γ (8, 29). Moreover, T cells that lack the IL-27 receptor expanded better even during resting conditions as well as Ag-mediated immune responses. It is possible that different IL-27 levels might explain different action of IL-27 on T cells (43); low IL-27 produced within noninflammatory conditions might result in antiproliferative function, whereas high IL-27 produced under inflammatory conditions may favor Th1 development. Consistently, IL-27R−/− CD4 T cells proliferate better following in vitro activation (21). Likewise, CD4 T cells from IL-27R−/− mice infected with Toxoplasma gondii expanded better, and their IL-2 production was also higher (44). IL-2 expression in activated T cells was directly suppressed by IL-27 in vitro (21). In support of this, IL-27R−/− OT-II CD4 T cells expanded greater when immunized in vivo with OVA peptide. Although the mechanism by which IL-27 mediates antiproliferative functions needs further examination, suppressor of cytokine signaling 3 induced by IL-27 may be involved in inhibiting T cell proliferation possibly via cyclin-dependent kinase inhibitor p27kip1 (21, 45).

Although IL-27 appears to be the major mediator regulating naïve T cell proliferation, it is unclear why continued presence of memory CD4 T cells is necessary to mediate the inhibition. The level of IL-27 expressed in vivo may be very low, and the t1/2 of IL-27 could be short (46). Thus, the action of IL-27 may be very local, unlike inflammatory cytokines that permeate an active lymph node and signal the majority of cells therein (47). Likewise, IL-2 needed for secondary expansion of memory CD8 T cells was reported to be of local autocrine origin (48). Whether IL-27 is the sole mediator or whether additional factors are involved depending on the type of responses will require further investigation. We believe that regulatory T cells are not involved in the mode of homeostatic regulation reported in this study, as we observed efficient IL-27 expression even after transfer of CD25−/− CD4 T cells (data not shown). Moreover, when TCRβ−/− mice that received 1′ CD25−/− CD4 T cells were treated with anti-CD25 (PC61) or control Ab at the time of 2′ naïve CD4 T cell transfer, the proliferation of newly transferred naïve CD4 T cells was efficiently inhibited by the 1′ memory CD4 T cells regardless of PC61 Ab treatment (data not shown), strongly suggesting that inducible regulatory T cells, which might have been generated from the 1′ transfer, do not appear to be involved in the inhibition process.

Selective expression of IL-27 in CD8+ DC subsets suggests that the capacity of DC to stimulate naïve T cell proliferation via a homeostatic mechanism might be divergent depending on the subsets (15, 49). In fact, it was demonstrated that CD8+ DC result in reduced proliferative T cell responses, when compared with CD8− DC (50), which is possibly mediated by IL-27. This mode of regulation under noninflammatory settings could be important and efficient in preventing unnecessary activation of naïve T cells during homeostatic control (23), consistent with the idea that CD8+ DC are primarily involved in peripheral tolerance (28).

There are some outstanding questions that need further investigation. We previously reported that TCR repertoire diversity is critical in regulating naïve T cell homeostasis because memory T cells with single or limited specificity were unable to inhibit naïve cell proliferation, whereas those cells with highly diverse specificity were fully capable of doing so (12). Thus, memory CD4 T cell interaction with DC and subsequent induction of IL-27 may be affected by the TCR repertoire complexity. It will be critical to understand how memory T cell repertoire complexity regulates DC production of IL-27. Moreover, in this study, we mainly studied homeostatic mechanism controlled by memory CD4 T cells. Whether memory CD8 T cells exert similar regulatory mechanisms, and, if so, whether IL-27 is also involved in the process needs to be tested.

In summary, homeostatic regulation of T cell proliferation is achieved by multiple mechanisms. IL-27 is mainly involved in limiting naïve T cell proliferation without exogenous Ag, whereas IL-7 might be primarily involved in regulating the overall size as well as the survival of the peripheral T cells. Concerted action between two cytokines and between different T cell subsets and DC is likely to contribute to T cell homeostasis. As dysregulated DC...
functions are likely to be associated with uncontrolled activation of T cells (51), controlling DC functions and naive T cell responses by memory CD4 T cells may represent a novel mechanism by which the immune system achieves a homeostatic balance in vivo.

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Disclosures

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References


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Supplementary Figure S1. Experimental Strategy

3 x 10^6 1' CD4 T cells: These cells undergo proliferation and differentiate into CD44\textsuperscript{high} memory CD4 T cells (ref. 4). These memory cells persist for long period of time. The total cellularity of the resulting memory T cells is only ~2 x 10^6 (ref. 4).

1 x 10^6 2' T cells: FACS sorted CFSE labeled Thy1.1 naïve T cells (CD4 + CD8) are then transferred into the same recipients that already received the 1' CD4 T cells 4 weeks earlier.

Control group of mice receive 2' T cells alone without the 1' CD4 transfer.

Mice are sacrificed 7 days post 2' transfer and CFSE profiles are examined.

Supplementary Figure S1: Experimental strategy
Supplementary Figure S2. Generation of BM chimeras that express different MHC II molecules
Groups of lethally irradiated B6xB10.A F1 Rag2/- mice received either B6xB10.A F1 Rag-/- BM cells or 1:1 mixture of B6 Rag-/- and B10.A Rag-/- BM cells. The former chimeras are expected to have antigen presenting cells that express both B6-derived I-A^b and B10.A-derived I-A^k/I-E^k on the same cells, while the latter chimeras are expected to have antigen presenting cells that express either I-A^b or I-A^k/I-E^k but not both on the cells.
Supplementary Figure S3. Generation of donor CD4 T cells and a proof-of-principle experiment

(A) Ly5.1 B10.A and Thy1.1 B6 BM cells were transferred into irradiated Ly5.2 B6 Rag-/- and Thy1.2 B10.A Rag-/- recipients, respectively. Naïve CD4 T cells were isolated by FACS 6 weeks after reconstitution and used as donor CD4 T cells. (B) A proof-of-principle experiment. I-A^k/I-E^k-restricted CD4 T cells were isolated from Thy1.2 B10.A Rag-/- recipients reconstituted with Thy1.1 B6 BM cells, and transferred into B6 Rag-/- or B10.A Rag-/- recipients. CD4 T cell proliferation was examined by CFSE dilution 7 days post transfer.
Supplementary Figure S4: Enhanced in vivo expansion of IL-27R-/记忆 T cells

Groups of irradiated Rag-/ mice were reconstituted with 1:1 mixture of Ly5.1 WT plus Ly5.2 IL-27R-/ (or Ly5.2 WT) BM cells. Total numbers of memory T cells were enumerated by FACS analysis. Each symbol represents individually tested mouse.