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p27Kip1 Negatively Regulates the Magnitude and Persistence of CD4 T Cell Memory

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Much is known about the differentiation of naive T cells into distinct lineages of effector cells, but the molecular mechanisms underlying the generation and maintenance of CD4 T cell memory are poorly characterized. Our studies ascribe a novel role for the cell cycle regulator p27Kip1 as a prominent negative regulator of the establishment and long-term maintenance of Th1 CD4 T cell memory. We demonstrate that p27Kip1 might restrict the differentiation and survival of memory precursors by increasing the T-bet/Bcl-6 ratio in effector CD4 T cells. By promoting apoptosis and contraction of effector CD4 T cells by mechanisms that are at least in part T cell intrinsic, p27Kip1 markedly limits the abundance of memory CD4 T cells. Furthermore, we causally link p27Kip1-dependent apoptosis to the decay of CD4 T cell memory, possibly by repressing the expression of γ-chain receptors and the downstream effector of the Wnt/β-catenin signaling pathway, Tcf-1. We extend these findings by showing that the antagonistic effects of p27Kip1 on CD4 T cell memory require its cyclin-dependent kinase-binding domain. Collectively, these findings provide key insights into the mechanisms underlying the governance of peripheral CD4 T cell homeostasis and identify p27Kip1 as a target to enhance vaccine-induced CD4 T cell memory. The Journal of Immunology, 2012, 189: 5119–5128.

CD4 T cells play a central role in orchestrating several facets of the anti-microbial immune response including macrophage-mediated defense, Ab production by B cells, activation and expansion of CD8 T cells, programming of CD8 T cell memory and maintenance of CD8 T cell responses during chronic viral infections (1, 2). In response to TCR and costimulatory signals, CD4 T cells clonally expand and differentiate into distinct effector cell types, including Th1, Th2, Th9, Th17, and T follicular helper (Tfh) cells, depending on the type of infection and lineage-specifying signals delivered to the responding T cells early in the infection (3). Typically, acute viral and intracellular bacterial infections stimulate a Th1 and Tfh effector response (4, 5). Following clearance of the pathogen, most effector CD4 T cells are lost, but a fraction of these cells that are programmed to survive differentiate into memory cells, which subsequently decline with an estimated half-life of 50–100 d (4–7). However, the mechanisms that control the differentiation or the attrition of memory CD4 T cells are not well understood.

Understanding the differentiation of effector and memory CD4 T cells is a highly active area of investigation. Recent studies have provided important insights on the lineage and differentiation of effector and memory CD4 T cells during Th1 responses (4, 5, 8–11). According to the current axiom, Ag-activated CD4 T cells differentiate into terminal Th1 effectors, memory precursor Th1 effectors, or Tfh cells, and the differentiation pathway is guided by exposure to cytokines such as IL-12 and IL-2, which control the expression of key transcription factors T-bet, Bcl-6, and BLIMP (11–13). It is noteworthy that apart from Th1 memory precursors, Tfh cells can also differentiate into conventional memory cells that can give rise to Th1 effectors and Tfh cells upon Ag re-exposure (8). Maintenance of a threshold number of memory CD4 T cells is critical for durable protective immunity, and the abundance of memory CD4 T cells at a given time point after immunization is a function of the initial clonal burst size of the memory precursors and the decay rate of differentiated memory cells (6, 13). Conceivably, during the clonal expansion phase of the T cell response, the proliferation rate exceeds apoptosis and, during memory attrition, cell death surpasses the rate of homeostatic proliferation (13). Thus, a dynamic balance between proliferation and apoptosis at different phases of the T cell response governs the magnitude and duration of CD4 T cell memory. However, the molecular mechanisms that govern cell cycle status and apoptosis of CD4 T cells from the context of CD4 T cell memory are poorly understood. Furthermore, it is widely accepted that intricate control of cell cycle entry and exit is integral and intimately linked to terminal differentiation and key cell fate decisions in many cell lineages (14–18), but the role of cell cycle regulators in orchestrating the differentiation program of effector and memory CD4 T cells is largely unknown.

Cellular proliferation is driven by kinase activity of cyclin/cyclin-dependent kinase (CDK) complexes, but the activity of CDKs is opposed by CDK inhibitors (CDKIs) (19). The CDKI p27Kip1 functions as an integral brake of the cell cycle by inhibiting cyclin/CDKs, especially the cyclin E/CDK2 complexes (20). The abundance of p27Kip1 is highest in quiescent cells, but it is rapidly downregulated during the G1 phase with low levels maintained through the S and G2/M phase of the cell cycle (21, 22). In addition to its prominent role as a negative regulator of the cell cycle, there is mounting evidence that p27Kip1 controls several cellular processes such as differentiation, cytokinesis, migration, transcription, and...
apoptosis (20, 23–25). In this study, we have unlocked unexpected antagonistic roles for p27Kip1 in the differentiation, establishment, and persistence of CD4 T cell memory following an acute lymphocytic choriomeningitis virus (LCMV) infection. Mechanistically, we demonstrate that p27Kip1 might promote the differentiation of terminal effector CD4 T cells at the expense of memory precursors by increasing the cellular levels of T-bet. Furthermore, not only did p27Kip1 markedly restrict the abundance of memory CD4 T cells, p27Kip1 was causally linked to apoptosis and erosion of CD4 T cell memory, possibly by negatively regulating the expression of γ-chain receptors and the downstream effector of the Wnt/β-catenin signaling pathway, Tcf-1. The inhibitory effects of p27Kip1 on CD4 T cell memory require the CDK-binding domain, and the p27Kip1-dependent loss of CD4 T cells occurs at least in part by T cell-intrinsic mechanisms. In summary, these findings have provided unexpected insights into the molecular mechanisms that regulate the establishment and persistence of CD4 T cell memory, which have significant implications in vaccine development.

Materials and Methods

Mice and viral infection

C57BL/6 mice were purchased from the National Cancer Institute or The Jackson Laboratory (Bar Harbor, ME). The p27+/− mice on a C57BL/6 background (26) were purchased from The Jackson Laboratory. The CDK−/− mice that express an amino-truncated mutant of p27Kip1 protein (amino acids 1–51 of p27Kip1 are deleted) were a gift from Dr. Andrew Koff (Memorial Sloan-Kettering Cancer Center, New York, NY) (27). Mice were infected i.p. with 2 × 10^5 PFU Armstrong strain of LCMV, and infectious LCMV was quantified by a plaque assay on Vero cells (28). All experiments were conducted in accordance with the approved protocols of the Institutional Animal Care and Use Committee.

Generation of bone marrow chimeras

Bone marrow cells (BMCs) were collected from bone marrow (BM) of wild-type (WT) and p27+/− mice with RPMI 1640 media. Single-cell suspensions of BMCs were depleted of T cells using anti-CD5 microbeads (Miltenyi Biotec, Auburn, CA). A 1:1 mixture of 7 × 10^6 T cell-depleted BMCs from WT (Ly5.1) and WT (Ly5.2) or p27+/− (Ly5.2) mice was adoptively transferred into lethally irradiated (900 rads) WT (Ly5.1) mice. Bone marrow-reconstituted WT Ly5.1 mice were treated with neomycin (0.025 mg/ml) and polynyxin B (0.013 mg/ml; Sigma-Aldrich, St. Louis, MO) in drinking water for up to 5 wk. Reconstitution of the lymphoid system by the donor BMCs was assessed at 5 wk and mice were infected with LCMV 7 wk after cell transfer.

Flow cytometry

Single-cell suspensions of mononuclear cells from spleen or peripheral blood were prepared by standard procedures. Splenocytes were stained with I-A^b/PHe6.7 MHC class II tetramers (provided by the National Institutes of Health Tetramer Core Facility, Emory University, Atlanta, GA) at 37 °C for 2 h, followed by staining for cell surface CD4. In some experiments, cells were costained with anti-CD44, anti-LFA-1, anti-CD62L, anti-CD122, anti-CD127, anti-Ly6C, and anti–PSGL-1 Abs. For intracellular cytokine staining, splenocytes were stimulated ex vivo with the p66 LCMV epitope peptide in the presence of brefeldin A for 5 h. After culture, cells were stained for surface and intracellular IFN-γ, TNF-α, and IL-2 using a Cytofix/Cytoperm intracellular staining kit (BD Biosciences, Franklin Lakes, NJ). Ki-67 staining was performed as previously described (29). Briefly, after surface staining, cells were fixed, permeabilized, and incubated with Abs against Ki-67 (BD Biosciences) for 45 min at room temperature. Staining for T-bet, Bcl-6, Bcl-2, Bcl-xL, and phosho-mammalian target of rapamycin (mTOR) were conducted using the phospho-staining protocol as previously described (30). Briefly, following surface staining, cells were fixed, lysed, and washed using a PhosFlow kit (BD Biosciences, San Jose, CA). Cells were subsequently stained with the specific Abs or isotype control Abs. All Abs were purchased from BD Biosciences (San Jose, CA), eBioscience (San Diego, CA), or Cell Signaling Technology (Danvers, MA).

Annexin V staining

At days 8 and 70 postinfecção (p.i.), splenocytes from WT and p27+/− mice were isolated and stained with anti-CD4 and MHC class II tetramer. Subsequently, annexin V staining (BD Biosciences, Franklin Lakes, NJ) was performed as previously described (29). The percentage of annexin V^high cells among virus-specific CD4 T cells was determined by flow cytometry.

BrdU staining

LCMV-immune mice were administered BrdU once i.p. and subsequently in drinking water for 12 d as described before (29). On day 13 after the initiation of BrdU administration, splenocytes were stained with anti-CD4, anti-CD62L, and I-A^b/gp66 tetramers. Subsequently, cells were stained for BrdU using a BrdU staining kit (BD Biosciences) according to the manufacturer’s recommendations.

Statistical analysis

Data were analyzed using Microsoft Excel. The p values were determined by a two-tailed Student t test, and significance was defined at p < 0.05.

Results

Homeostasis of CD4 T cells in p27Kip1-deficient mice

To evaluate whether loss of p27Kip1 affected the basal homeostasis of the peripheral CD4 T cell compartment, we compared the numbers and the activation status of CD4 T cells in WT and p27Kip1-deficient (p27−/−) mice at 6–8 wk age. Whereas the percentages of CD44hi (activated/memory) cells among CD4 T cells were slightly reduced, the total numbers of CD44hi CD4 T cells were not significantly altered in spleens of p27−/− mice (Fig. 1A, 1B). However, there were significantly more CD44hi (naive) CD4 T cells (∼1.7-fold) in spleens of p27−/− mice as compared with those in WT mice (Fig. 1B). Next, we assessed the effect of p27Kip1 deficiency on the proliferation of naive and activated/memory CD4 T cells. Fig. 1C shows that the percentages of Ki-67+ cells among naive or activated/memory CD4 T cells in p27−/− mice were higher than in WT mice. These data suggested that p27Kip1 regulates the homeostasis of CD4 T cells.

FIGURE 1. Homeostasis of mature CD4 T cells in p27−/− mice. Splenocytes from 6- to 8-wk-old WT and p27−/− mice were stained with anti-CD4, anti-CD44, and anti-Ki-67 Abs. (A and B) The percentages of naive (CD44lo) or activated/memory (CD44hi) CD4 T cells were quantified by flow cytometry. (C) The percentage of Ki-67+ cells among naive or activated/memory CD4 T cells was assessed by flow cytometry. Data are from four mice per group. *p < 0.05.
CDKI p27Kip1 restricts the primary expansion of CD4 T cells during an acute LCMV infection

To determine whether p27Kip1 regulates the primary CD4 T cell response to an acute viral infection, cohorts of WT and p27−/− mice were infected with LCMV. At day 8 p.i., the number of activated (CD44hi) and I-Aβ-restricted LCMV epitope gp66-specific CD4 T cells were quantified by flow cytometry. The numbers of activated CD4 T cells in spleens of p27−/− mice were substantially greater (~200%) than in WT mice (Fig. 2A). The percentages of gp66-specific CD4 T cells were not affected by p27Kip1 deficiency, but the total numbers of these cells in spleens of p27−/− mice were ~2-fold higher than in WT mice (Fig. 2B). Increased numbers of gp66-specific CD4 T cells resulted from larger spleen size in p27−/− mice. However, gp66-specific CD4 T cells from both WT and p27−/− mice displayed the expected CD44hi/CD62Llo/LFA-1hi activated/effector phenotype with no significant differences in the cell surface expression of cytokine receptors CD127 or CD122 (Fig. 2C). Next, we assessed the effector functions of gp66-specific CD4 T cells by quantifying Ag-triggered production of IFN-γ, IL-2, and TNF-α. The gp66-specific CD4 T cells from WT and p27−/− mice produced comparable levels of IFN-γ, and the percentages of these cells that produced IL-2 and TNF-α were unaffected by p27Kip1 deficiency (Fig. 2D, 2E). Collectively, these results suggested that p27Kip1 limited the accumulation of Ag-specific CD4 T cells without affecting their effector function or cell surface phenotype during an acute viral infection. Note that infectious LCMV was below the levels of detection in both WT and p27−/− mice at 8 d p.i., which is consistent with normal CD8 T cell responses in the absence of p27Kip1 (31).

CDKI p27Kip1 regulates the proliferation and apoptosis of gp66-specific CD4 T cells

To discern whether p27Kip1 limited the accumulation of CD4 T cells by its antiproliferative function, we quantified the percentages of Ki-67+ gp66-specific CD4 T cells at day 8 p.i. The percentages of proliferating gp66-specific CD4 T cells were significantly higher in p27−/− mice than in WT mice (Fig. 3A, 3B). Furthermore, the mean fluorescence intensity (MFI) for Ki-67 staining in Ki-67+ p27−/− CD4 T cells was greater than in WT CD4 T cells (Fig. 3A). To evaluate the possibility that p27Kip1 also exerted proapoptotic effects on gp66-specific CD4 T cells, we measured annexin V binding directly ex vivo. Surprisingly, the percentages of annexin Vhi gp66-specific CD4 T cells were significantly lower in p27−/− mice than in WT mice (Fig. 3C). However, the observed reduction in annexin V binding to p27−/−/gp66-specific CD4 T cells cannot be explained by alterations in the cellular levels of the antiapoptotic molecules Bcl-2 or Bcl-xL (data not shown). Nevertheless, data in Fig. 3 collectively illustrated that the growth inhibitory function of p27Kip1 in gp66-specific CD4 T cells might include both antiproliferative and proapoptotic effects.

CDKI p27Kip1 regulates T-bet expression and differentiation of effector CD4 T cells

Studies by Kaech and colleagues (4) have suggested that the heterogeneous population of virus-specific effector CD4 T cells present at day 8 after LCMV infection can be classified into three subsets based on differential expression of PSGL-1, Ly6C, and T-bet: PSGL-1hiLy6chiT-bethi, PSGL-1hiLy6cliT-betint, and PSGL-1loLy6clT-bethi subsets. Whereas the aforementioned subsets were described based on studies that used SMARTA TCR transgenic CD4 T cells, our studies on polyclonal CD4 T cells identified three subsets, namely PSGL-1hiLy6chi, PSGL-1loLy6clhi, and PSGL-1loLy6cllo (Fig. 4A). However, in agreement with...
Marshall et al. (4) the levels of T-bet expression correlated with Ly6C expression in our studies (data not shown). Next, we explored whether enhanced accumulation of CD4 T cells induced by p27<sup>Kip1</sup> deficiency accompanied altered CD4 T cell differentiation following LCMV infection (Fig. 4). Strikingly, there was an impressive increase in the percentages of the PSGL-1<sup>hi</sup>Ly6C<sup>lo</sup> subset of gp66-specific CD4 T cells in spleen of p27<sup>/−</sup> mice as compared with those in WT mice (Fig. 4A, 4B); the increase in the PSGL-1<sup>hi</sup>Ly6C<sup>lo</sup> subset in p27<sup>/−</sup> mice occurred at the expense of the other two Ly6C<sup>hi</sup> subsets, and note that cells with a memory-like transcription profile are enriched in the Ly6C<sup>lo</sup> subset of CD4 T cells (4). To assess whether the enrichment for the PSGL-1<sup>lo</sup>Ly6C<sup>lo</sup> CD8 T cells in p27<sup>/−</sup> mice was driven by enhanced proliferation of this subset, we compared Ki-67 expression in the aforementioned three subsets of gp66-specific CD4 T cells at day 8 p.i. We observed that p27<sup>Kip1</sup> deficiency caused a consistent increase in the percentages of proliferating cells in all three subsets (Fig. 4C). Therefore, differential proliferation did not sufficiently explain the selective enrichment for the PSGL-1<sup>lo</sup>Ly6C<sup>lo</sup> CD4 T cell subset in p27<sup>/−</sup> mice.

It is well established that the transcription factor T-bet promotes the differentiation of Th1 cells, but there is good evidence that T-bet also drives the terminal differentiation of Th1 effector T cells (4, 5, 12). Specifically, the balance of T-bet and Bcl-6 appears to govern the differentiation program of Ag-activated T cells (i.e., Th1 terminal effectors) versus T<sub>H</sub>Th cells and/or Th1 memory precursors (9). To gain insight into how p27<sup>Kip1</sup> might regulate differentiation of CD4 T cells, we quantified T-bet and Bcl-6 expression in gp66-specific CD4 T cells at day 8 p.i. Interestingly, there was a prominent reduction in the abundance of T-bet but not Bcl-6 in gp66-specific CD4 T cells from p27<sup>/−</sup> mice as compared with those in their WT counterparts (Fig. 4D, 4E). The reduction in T-bet expression in p27<sup>/−</sup> gp66-specific CD4 T cells did not result from enrichment for the T-bet<sup>lo</sup>/Ly6C<sup>lo</sup> population because T-bet expression was significantly reduced in both Ly6C<sup>hi</sup> and Ly6C<sup>lo</sup> CD4 T cells in p27<sup>/−</sup> mice (data not shown). We propose that the diminished T-bet/Bcl-6 ratio (Fig. 4F) might have favored the differentiation of Ly6C<sup>lo</sup> memory precursor CD4 T cells at the expense of Ly6C<sup>hi</sup> terminal Th1 effector cells in p27<sup>/−</sup> mice.

In CD4 T cells, upregulation of T-bet expression and differentiation into Th1 effectors requires phosphorylation of the mTOR by upstream kinases such as Akt (32). To evaluate whether reduced T-bet expression was related to lower activation of mTOR in p27<sup>/−</sup>- CD4 T cells, we quantified phosphorylation of mTOR in naive
(CD44hi), activated (CD44hi), and gp66-specific CD4 T cells at day 8 p.i. The phosphorylation of mTOR (measured by the MFI) in CD44hi cells and gp66-specific CD4 T cells was greater than in CD44hi cells in both WT and p27−/− mice (data not shown). However, the phosphorylation of gp66-specific CD4 T cells from p27−/− mice was not significantly different as compared with their WT counterparts (data not shown). Thus, reduced levels of T-bet in p27−/− CD4 T cells was not coupled with lower mTOR activation, at least at day 8 p.i.

**Reduced contraction of effector CD4 T cells in the absence of p27Kip1**

Peak numbers of gp66-specific CD4 T cells are attained at day 8 p.i., and in the ensuing 2–3 wk, a large fraction of the expanded CD4 T cells contract, presumably by apoptosis (4). The remainder of the cells that survive the contraction differentiate into memory T cells. Following LCMV infection, the contraction kinetics for WT and p27-deficient CD4 T cells were noticeably different (Fig. 5A, 5B). Between days 8 and 15 p.i., a significant number (~70%; p = 0.003) of the gp66-specific CD4 T cells were lost in the WT mice. In contrast, the numbers of gp66-specific CD4 T cells in p27−/− mice showed a modest drop (not statistically significant) between days 8 and 15 p.i. By virtue of diminished contraction in p27−/− mice, the differences in the numbers of gp66-specific CD4 T cells between WT and p27−/− mice (~450%) were further magnified at day 15 p.i. as compared with day 8 p.i. It is likely that both increased proliferation and reduced apoptosis (Fig. 3) contributed to diminished contraction of CD4 T cells in p27−/− mice. We propose that reduced apoptosis and contraction of gp66-specific p27−/− CD4 T cells might be linked to aneliorated terminal differentiation consequential to lower levels of T-bet.

**CDKI p27Kip1 constrains the magnitude and maintenance of CD4 T cell memory**

We further examined the role of p27Kip1 in regulating the homeostasis of memory CD4 T cells in LCMV-immune mice. To analyze whether p27Kip1 regulated the abundance of CD4 T cell memory, we enumerated gp66-specific CD4 T cells at days 50 and 165 p.i. (Fig. 6). At day 50 p.i., the frequencies and total number of gp66-specific memory CD4 T cells in spleen of p27−/− mice were markedly greater than in WT mice. Remarkably, with time, these differences were further magnified, and by day 165 p.i. there were ~12-fold more gp66-specific memory CD4 T cells in p27−/− mice than in their WT counterparts (Fig. 6A). However, p27Kip1 deficiency did not affect the differentiation of central and effector memory subsets (data not shown), which suggested that increased abundance of memory CD4 T cells in p27−/− mice might not be linked to differences in TCR signaling during the primary response and/or clonal abundance in the naive TCR repertoire (13). This is because an increase in the clonal abundance in the naive T cell repertoire and/or dampened TCR signaling in p27−/− mice would be expected to skew the T cells toward the central memory phenotype (13). Additionally, loss of p27Kip1 did not affect the expression of adhesion molecules CD44 and LFA-1 on memory CD4 T cells (data not shown). As a qualitative measure of memory CD4 T cells, we compared the cytokine-producing ability of memory CD4 T cells from WT and p27−/− mice (Fig. 6B). At both days 50 and 165 p.i., 70–80% of gp66-specific IFN-γ-producing CD4 T cells from WT and p27−/− mice also produced TNF-α and IL-2 (Fig. 6B). However, the total numbers of these triple cytokine-producing CD4 T cells in p27−/− mice were significantly higher than in WT mice (Fig. 6B). Collectively, results in Fig. 6 showed that p27Kip1 deficiency increased the abundance of memory CD4 T cells without affecting the subset distribution or their quality.

**Acute viral infections typically induce potent CD4 and CD8 T cell memory**

Whereas a numerically stable pool of memory CD8 T cells is maintained for extended periods of time, CD4 T cell memory is less stable (5, 7). CD4 T cell memory shows a continuous decline over time with a calculated half-life of 50–100 d (5–7). The molecular mechanisms underlying the erosion of CD4 T cell memory are largely unknown. To evaluate whether p27Kip1 regulated the maintenance of memory CD4 T cells, we performed a detailed kinetic assessment of gp66-specific CD4 T cells between days 8 and 300 p.i. (Fig. 7A). Consistent with published work (7, 33), gp66-specific CD4 T cells in WT mice showed a continuous decline after day 8 p.i. in three arbitrarily defined phases: 1) the steep contraction between days 8 and 15 p.i.; 2) an ~2-fold decline between days 15 and 70 p.i.; and 3) a further 3.5- to 5-fold decline between days 70 and 300 p.i. Unexpectedly, in the p27−/− mice, there was only one phase of decline between days 8 and 51 p.i. when the number of gp66-specific CD4 T cells dropped by ~2.4-fold. Remarkably, after the day 51 p.i. time point, we did not detect significant attrition of memory CD4 T cells in p27−/− mice for at least until day 300 p.i. (Fig. 7A). These data strongly implicated a role for p27Kip1 in the erosion of memory CD4 T cells in LCMV-immune mice.

An imbalance in the apoptosis and homeostatic proliferation is thought to underlie the decline of memory CD4 T cells in WT mice (13). Therefore, the stability of CD4 T cell memory in p27−/− mice could result from increased homeostatic proliferation and/or by reduced apoptosis. To examine these possibilities, we quantified proliferation of gp66-specific CD4 T cells in LCMV-immune WT and p27−/− mice by staining for Ki-67 and measuring BrdU incorporation in vivo. At days 70 (data not shown) and 90 p.i., the percentages of Ki-67+ or BrdU+ gp66-specific CD4 T cells in p27−/− mice were similar to those in WT mice (Fig. 7B), which suggested that altered proliferation did not explain improved sustenance of CD4 T cell memory in p27−/− mice. Similar proliferation profiles of WT and p27−/− gp66-specific memory CD4 T cells were also observed at day 165 p.i. (data not shown). To determine whether p27Kip1 regulated the steady-state apoptosis of gp66-specific memory CD4 T cells, we measured annexin V binding at day 70 p.i. (Fig. 7C). The percentages of annexin V+ gp66-specific memory CD4 T cells in p27−/− mice were significantly lower than in WT mice. These data suggested that p27Kip1 might contribute to the decay of CD4 T cell memory by promoting apoptosis and not by suppressing proliferation.

![FIGURE 5. CDKI p27Kip1 regulates early contraction of effector CD4 T cells following acute LCMV infection. (A) WT and p27−/− mice were infected with LCMV and at the indicated days p.i., gp66-specific CD4 T cells were quantified in spleens. (B) To evaluate the difference in contraction kinetics of gp66-specific CD4 T cells in WT and p27−/− mice, we calculated the fold drop in the number of gp66-specific CD4 T cells between days 8 and 15 or days 15 and 30 p.i. Data are representative of three to four independent experiments with three to six mice per group per experiment for each indicated time point. Error bars represent the SEM. *p < 0.05.](http://www.jimmunol.org/doi/pdf/10.4049/jimmunol.1101082)
Maintenance of CD4 T cell memory depends on signaling via the TCR and the γ-chain cytokine receptors, especially CD127, the IL-7 receptor (6, 13, 34, 35). To begin to understand the mechanisms underlying the maintenance of memory CD4 T cells in p27<sup>Kip1</sup>−/− mice, we compared expression of the IL-7 receptor on WT and p27<sup>Kip1</sup>−/− activated/memory CD4 T cells in LCMV-immune mice. Interestingly, the percentages of IL-7R<sup>hi</sup> activated/memory CD4 T cells were significantly higher in p27<sup>Kip1</sup>−/− mice as compared with those in WT mice (Fig. 7D). Because higher expressions of Tcf-1 and Bcl-2 are known to promote survival of memory T cells (36), and p27<sup>Kip1</sup> can suppress cellular Tcf levels (37), we compared the levels of these proteins in WT and p27<sup>Kip1</sup>−/− memory CD4 T cells (Fig. 7E). The expression of Tcf-1 but not Bcl-2 in p27<sup>Kip1</sup>−/− gp66-specific memory CD4 T cells was higher than in WT memory CD4 T cells. Thus, the stability of CD4 T cell memory in p27<sup>Kip1</sup>−/− mice was associated with increased expression of IL-7 receptors and Tcf-1, which might promote cell survival.

**T cell-intrinsic regulatory mechanisms of p27<sup>Kip1</sup>**

Data presented thus far have clearly demonstrated a role for p27<sup>Kip1</sup> in governing the establishment and maintenance of CD4 T cell memory. To ascertain whether regulation of memory CD4 T cell homeostasis by p27<sup>Kip1</sup> occurred by T cell-intrinsic mechanisms, two types of bone marrow chimeric mice were constructed using congenic Ly5.1 and Ly5.2 mice: 1) control chimeras (CCs) were generated by reconstituting lethally irradiated WT/Ly5.1 mice with bone marrow cells from WT/Ly5.1 and WT/Ly5.2 mice; and 2) experimental chimeras (ECs) were derived by reconstituting lethally irradiated WT/Ly5.1 mice with bone marrow cells from WT/Ly5.1 and p27<sup>Kip1</sup>−/−/Ly5.2 mice. Seven weeks after bone marrow reconstitution, CC and EC mice were infected with LCMV and the responses of WT and p27<sup>Kip1</sup>−/− CD4 T cells were examined in the spleen at day 8 p.i. As shown in Fig. 8A, the percentages of WT/Ly5.1 and WT/Ly5.2 gp66-specific CD4 T cells in spleen were comparable in CCs and ECs at day 8 p.i. However, the percentages of p27<sup>Kip1</sup>−/−/Ly5.2 gp66-specific CD4 T cells were slightly but statistically higher than their WT/Ly5.1 counterparts. Based on these results and data from Fig. 2 we inferred that p27<sup>Kip1</sup>-mediated T cell-intrinsic effects might play a small but significant role in regulating the primary expansion of CD4 T cells during an acute LCMV infection. To further elucidate whether p27<sup>Kip1</sup> regulated the CD4 T cell contraction and early memory by T cell-intrinsic mechanisms, we quantified gp66-specific CD4 T cells in spleen at day 45 p.i. (Fig. 8B). In the CCs and ECs, the percentages of WT/Ly5.1 or WT/Ly5.2 gp66-specific CD4 T cells in spleen were comparable in CCs and ECs at day 8 p.i. However, the percentages of p27<sup>Kip1</sup>−/−/Ly5.2 gp66-specific CD4 T cells in ECs were slightly but statistically higher than their WT/Ly5.1 counterparts. Based on these results and data from Fig. 2 we inferred that p27<sup>Kip1</sup>-mediated T cell-intrinsic effects might play a small but significant role in regulating the primary expansion of CD4 T cells during an acute LCMV infection. To further elucidate whether p27<sup>Kip1</sup> regulated the CD4 T cell contraction and early memory by T cell-intrinsic mechanisms, we quantified gp66-specific CD4 T cells in the spleen at day 45 p.i. (Fig. 8B). In the CCs and ECs, the percentages of WT/Ly5.1 or WT/Ly5.2 gp66-specific CD4 T cells were ~1.5%, whereas the percentages of p27<sup>Kip1</sup>−/−/Ly5.2 gp66-specific CD4 T cells were ~3.8%. Whereas WT/Ly5.1 and WT/Ly5.2 CD4 T cells contracted by 4.5- to 6.5-
fold between days 8 and 45 p.i., p27−/− mice were collected at the indicated time points, and the numbers of gp66-specific CD4 T cells were determined by intracellular cytokine staining for IFN-γ. Data are from two to four independent experiments with three to six mice per group per experiment. Error bars represent the SEM. *p < 0.05. (B) At day 90 p.i., mice were administered BrdU for 12 d in drinking water. Splenocytes from WT and p27−/− mice were stained with anti-CD4, I-A^b gp66 tetramer, and anti−Ki-67 or anti-BrdU Abs. Data are expressed as percentage of Ki-67^+ or BrdU^+ cells among gp66-specific CD4 T cells. (C) At day 70 p.i., splenocytes were stained with anti-CD4, I-A^b gp66 tetramer, and annexin V directly ex vivo. Data are expressed as the percentage of annexin V^+ cells among gp66-specific CD4 T cells. (D) Splenocytes from WT and p27−/− mice were stained with anti-CD127, anti-CD4, and anti-CD44, and the graph shows the percentage of CD127^+ activated/memory CD4 T cells. (E) Following staining with anti-CD4 and I-A^b gp66 tetramer, intracellular staining for either anti−Tcf-1 or anti−Bcl-2 was performed directly ex vivo. Graphs represent the ΔMFI (observed MFI minus isotype control MFI) with Tcf-1 (left) and Bcl-2 (right) for gp66-specific CD4 T cells. Data are from at least two independent experiments with three to six mice per group per experiment. Error bars represent the SEM. *p < 0.05.

The CDK-binding domain of p27Kip1 constrains CD4 T cell memory

Apart from its widely accepted cyclin/CDK inhibitory function, p27Kip1 also promotes oncogenesis and regulates cellular activities such as cytokinesis, cell migration, and transcription by mechanisms independent of its CDK-binding domain (20, 23–25). To determine whether p27Kip1 regulated CD4 T cell homeostasis by mechanisms independent of its cyclin/CDK regulatory function, we used p27 mutant mice (CDK−/−) in which the truncated p27Kip1 is unable to interact with and inhibit cyclins and CDKs (27). Virus-specific CD4 T cell responses were quantified at day 8 p.i. after infecting WT and CDK−/− mice with LCMV (Fig. 9A–C). The numbers of activated and gp66-specific CD4 T cells in CDK−/− mice were significantly higher than in WT mice (Fig. 9A, 9B). Likewise, the numbers of functional gp66-specific CD4 T cells that produced IFN-γ or IFN-γ plus TNF-α and IL-2 were significantly increased in CDK−/− mice (Fig. 9C). We found that the augmented accumulation of gp66-specific CD4 T cells in CDK−/− mice was associated with increased cellular proliferation (Fig. 9D). Thus, the restriction of the abundance of activated CD4 T cells by p27 Kip1 requires the CDK-binding domain.

Next, we examined whether p27Kip1 regulated the differentiation of gp66-specific CD4 T cells via its CDK-binding domain. Similar to our finding with the p27−/− mice (Fig. 4), the percentages of
FIGURE 9. A role for the CDK-binding domain of p27<sup>Kip1</sup> in regulating CD4 T cell memory. WT C57BL/6 or mice lacking the CDK-binding domain of p27<sup>Kip1</sup> (CDK<sup>-/-</sup>) were infected with LCMV. (A and B) At day 8 p.i., the numbers of activated (CD44<sup>hi</sup>) and gp66 tetramer<sup>+</sup> CD4 T cells were quantified by flow cytometry. (C) On day 8 p.i., splenocytes from WT and CDK<sup>-/-</sup> mice were stimulated with the gp66 peptide and the percentages IFN-g<sup>-</sup>, IL-2<sup>-</sup>, and TNF-α<sup>-</sup>-producing cells were assessed by intracellular cytokine staining. The total numbers of INF-γ- and triple cytokine-producing (IFN-γ<sup>-</sup>, IL-2<sup>-</sup>, and TNF-α<sup>-</sup>) gp66-specific CD4 T cells per spleen are shown. (D) At day 8 p.i., proliferation of gp66-specific CD4 T cells was determined by staining for Ki-67 directly ex vivo. On the left are the representative plots showing the percentages of Ki-67<sup>+</sup> cells among gp66-specific CD4 T cells or the MFI for Ki-67 in gp66-specific CD4 T cells. The bar graph on the right shows the average percentages of Ki-67<sup>+</sup> cells among gp66-specific CD4 T cells. (E) To quantify subsets of gp66-specific effector CD4 T cells at day 8 p.i., splenocytes were stained with anti-CD4, gp66 tetramer, anti–PSGL-1, and anti-Ly6C. Percentages of the indicated subsets among gp66-specific CD4 T cells were quantified by flow cytometry. (F) Bar graph shows the percentages of Ki-67<sup>+</sup> cells among the indicated subsets of gp66-specific effector CD4 T cells at day 8 p.i. Numbers in parentheses show percentage increase of p27<sup>Kip1</sup> (CDK) expression and therefore a Bcl-6 skewed T-bet/Bcl-6 ratio in effector CD4 T cells. (G) On day 8 p.i., levels of T-bet and Bcl-6 proteins in gp66-specific CD4 T cells were measured by flow cytometry. Graphs represent the SEM. *p < 0.05.

Data in Fig. 9H illustrate the differential kinetics of the CD4 T cell response to LCMV infection in WT and CDK<sup>-/-</sup> mice. The percent of CD127<sup>hi</sup> and CD122<sup>hi</sup> cells among gp66-specific memory CD4 T cells in spleens of WT and CDK<sup>-/-</sup> mice were determined by flow cytometry. (H) On day 8 p.i., proliferation of gp66-specific CD4 T cells was determined by staining for Ki-67 directly ex vivo. On the left are the representative plots showing the percentages of Ki-67<sup>+</sup> cells among gp66-specific CD4 T cells or the MFI for Ki-67 in gp66-specific CD4 T cells. The bar graph on the right shows the average percentages of Ki-67<sup>+</sup> cells among gp66-specific CD4 T cells. (I) At day 100 p.i., the percentages of proliferating cells among gp66-specific memory CD4 T cells were determined by staining for Ki-67. (J) At day 60 p.i., splenocytes from WT and CDK<sup>-/-</sup> mice were stained with anti-CD4, I-A<sup>b</sup> gp66 tetramer, and annexin V. Bar graph shows the percentages of annexin V<sup>+</sup> cells among gp66-specific memory CD4 T cells. (K) Bar graph shows the percentage of CD127<sup>hi</sup> and CD122<sup>hi</sup> cells among gp66-specific memory CD4 T cells in spleens of WT and CDK<sup>-/-</sup> mice on day 100 p.i. Data are representative of two to four independent experiments with three to six mice per group per experiment for each indicated time points. Error bars represent the SEM. *p < 0.05.
similar in WT and CDK\(^{-/-}\) mice (data not shown). These data reaffirm the inference that enhanced CD4 T cell memory was less likely to result from increased abundance of naive gp66-specific CD4 T cells or dampened TCR signaling in the absence of CDK-binding functions of p27\(^{kip1}\). In summary, studies in CDK\(^{-/-}\) mice (Fig. 9) fully recapitulated the "CD4 T cell memory phenotype" of p27\(^{-/-}\) mice (Figs. 2–7). This formed the basis for our inference that the regulation of establishment and maintenance of CD4 T cell memory occurs by cyclin/CDK-dependent functions of p27\(^{kip1}\).

**Discussion**

CD4 T cells are central cells of the adaptive immune system, and elicitation of potent CD4 T cell memory is key to the development of enduring B cell- or CD8 T cell-based protective immunity afforded by vaccines. The molecular mechanisms that govern the establishment and maintenance of CD4 T cell memory are not well understood. In this study, we have undertaken a comprehensive evaluation of the role of CDKI p27\(^{kip1}\) in regulating all facets of the CD4 T cell response to an acute viral infection. Collectively, data presented in this manuscript illuminate the functions of p27\(^{kip1}\) as a key negative regulator of the abundance of CD4 T cell memory. We document that p27\(^{kip1}\) limits the magnitude of CD4 T cell memory by 1) repressing the differentiation program of CD4 memory precursors, 2) antagonizing the survival of effector CD4 T cells during effector to memory transition, and 3) limiting the survival and long-term maintenance of memory CD4 T cells. These results are expected to have significant implications in development of vaccines that induce enduring immunity.

We find that loss of p27\(^{kip1}\) lead to increased accumulation of LCMV-specific CD4 T cells during the primary response to LCMV. Although p27\(^{kip1}\) is primarily known as a cell cycle inhibitor, we find that loss of p27\(^{kip1}\) not only increased the proliferation but also reduced the apoptosis of LCMV-specific CD4 T cells. The mechanisms underlying the regulation of apoptosis by p27\(^{kip1}\) during clonal expansion of CD4 T cells needs further investigation. Furthermore, we cannot exclude the possible contribution of the altered proliferation of naive CD4 T cells to the enhanced primary CD4 T cell response to LCMV in p27\(^{-/-}\) mice.

Our finding that p27\(^{kip1}\) deficiency affected the percentages of effector CD4 T cell subsets is unexpected and novel. More surprising is the finding that p27\(^{kip1}\) deficiency decreased the abundance of a canonical transcription factor T-bet in LCMV-specific effector cells. Lower T-bet levels in turn reduced the T-bet/Bcl-6 ratio, favoring the development of memory CD4 T cells at the expense of the terminal effector cells in p27\(^{-/-}\) mice. Mechanistically, it is possible that lower levels of T-bet/Bcl-6 complexes allowed T-bet–free Bcl-6 to repress BLIMP expression, thereby diminishing the BLIMP-driven terminal differentiation of effector cells (9). It is noteworthy that lower T-bet levels in CD4 T cells did not affect IFN-\(\gamma\) production in p27\(^{-/-}\) mice. Perhaps the reduced levels of T-bet in p27\(^{-/-}\) effectors are sufficient for inducing effector functions, and higher T-bet expression might be required to drive differentiation into terminal effectors. How might p27\(^{kip1}\) promote T-bet expression? T-bet expression in T cells is induced by cytokines such as IL-2 and IL-12 early in the response (3, 5). However, CD4 T cells from p27\(^{-/-}\) mice did not show alterations in IL-2 production or the expression of the high-affinity IL-2 receptor at day 5 p.i. (data not shown), nor did CD11c\(^{+}\) dendritic cells from p27\(^{-/-}\) mice differ from WT dendritic cells, pertaining to IL-12 gene expression at day 5 p.i. (data not shown), mTOR activation has been implicated in T-bet induction (32), but we did not find differences in the phosphorylation of mTOR between WT and p27\(^{-/-}\) CD4 T cells, at least at day 8 p.i. The use of Ly6C to differentiate terminal effectors from memory precursors is by no means absolute because both Ly6C\(^{hi}\) and Ly6C\(^{lo}\) effector CD4 T cells do undergo significant contraction, and it is likely that both subsets can give rise to memory cells (4). Although p27 deficiency did alter the relative proportions of Ly6C\(^{hi}\) and Ly6C\(^{lo}\) in favor of the Ly6C\(^{lo}\) cells, marked reduction in contraction of effectors and enhancement of CD4 T cell memory in p27\(^{-/-}\) mice might be consequential to reduced T-bet expression and failure to trigger the apoptotic program in both Ly6C\(^{hi}\) and Ly6C\(^{lo}\) effector subsets. Note that p27\(^{kip1}\) might regulate the survival of effector CD4 T cells at least in part by cell-intrinsic mechanisms because contraction of p27\(^{-/-}\) CD4 T cells was reduced in mixed bone marrow chimeras. Regardless, to our knowledge this is the first description of the involvement of p27\(^{kip1}\), a cell cycle regulator in effector differentiation and T-bet expression, which warrants further investigation to evaluate the underlying mechanisms.

Although p27\(^{kip1}\) is known primarily as a suppressor of cellular proliferation, our studies strongly implicate a proapoptotic function for p27\(^{kip1}\) in the contraction of effector CD4 T cells. We find that both proapoptotic and antiproliferative functions of p27\(^{kip1}\) require the CDK-binding domain and are at least in part T cell-intrinsic because p27\(^{-/-}\) CD4 T cells exhibited increased expansion and reduced contraction in mixed bone marrow chimeras. The reduced contraction of effector CD4 T cells in p27\(^{-/-}\) mice and enhancement in the abundance of memory cells did not result from increased expression of Bcl-2 or Bcl-x\(_L\). Instead, we propose that enhanced CD4 T cell memory in p27\(^{-/-}\) mice is linked to a less terminally differentiated state of effector CD4 T cells by virtue of decreased T-bet/Bcl-6 ratios. The precise molecular mechanisms underlying the T-bet–driven terminal differentiation and apoptosis of effector CD4 T cells are currently unknown.

Another remarkable discovery reported in this study is the identification of p27\(^{kip1}\)-dependent apoptosis, and not suppressed homeostatic proliferation, as a mechanism to explain the attrition of CD4 T cell memory. It has been reported that p27\(^{kip1}\) might induce apoptosis (25) by inhibiting the expression of Mcl-1 (38). However, we find no significant alterations in the levels of Bcl-2 or Mcl-1 (data not shown) in p27\(^{-/-}\) memory CD4 T cells. Instead, we find that the mechanisms underlying the proapoptotic effects of p27\(^{kip1}\) on memory CD4 T cells might include inhibition of the prosurvival Wnt/\(\beta\)-catenin pathway and repression of IL-7 and IL-15 receptor expression.

Although recent studies have shed light on the ontogeny and lineage of effector and memory CD4 T cells, the underlying mechanisms of differentiation are poorly understood. Tight control of cellular proliferation and apoptosis during clonal expansion, contraction, and memory phases of the T cell response governs the abundance and maintenance of memory CD4 T cells. Consistent with this idea, this study has provided unexpected insights into how a key cell cycle regulator, p27\(^{kip1}\), plays pivotal roles in restricting and enhancing the differentiation and maintenance of Th1 memory CD4 T cells. We ascribe a previously unknown role for p27\(^{kip1}\) as a critical negative regulator of CD4 T cell immunity and propose that p27\(^{kip1}\) is a strong candidate target for immunomodulation to enhance vaccine-induced protective immunity.

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**Disclosures**

The authors have no financial conflicts of interest.