T-cell intrinsic and extrinsic mechanisms of p27Kip1 in the regulation of CD8 T-cell memory

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CD8 T cells exhibit dynamic alterations in proliferation and apoptosis during various phases of the CD8 T-cell response, but the mechanisms that regulate cellular proliferation from the standpoint of CD8 T-cell memory are not well defined. The cyclin-dependent kinase inhibitor p27Kip1 functions as a negative regulator of the cell cycle in T cells, and it has been implicated in regulating cellular processes, including differentiation, transcription and migration. Here, we investigated whether p27Kip1 regulates CD8 T-cell memory by T-cell-intrinsic or T-cell-extrinsic mechanisms, by conditional ablation of p27Kip1 in T cells or non-T cells. Studies of T-cell responses to an acute viral infection show that p27Kip1 negatively regulates the proliferation of CD8 T cells by T-cell-intrinsic mechanisms. However, the enhanced proliferation of CD8 T cells induced by T-cell-specific p27Kip1 deficiency minimally affects the primary expansion or the magnitude of CD8 T-cell memory. Unexpectedly, p27Kip1 ablation in non-T cells markedly augmented the number of high-quality memory CD8 T cells by enhancing the accumulation of memory precursor effector cells without increasing their proliferation. Further studies show that p27Kip1 deficiency in immunizing dendritic cells fail to enhance CD8 T-cell memory. Nevertheless, we have delineated the T-cell-intrinsic, anti-proliferative activities of p27Kip1 in CD8 T cells from its role as a factor in non-T cells that restricts the development of CD8 T-cell memory. These findings have implications in vaccine development and understanding the mechanisms that maintain T-cell homeostasis.

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Encounter with mature, antigen-loaded dendritic cells (DCs) is the beginning of the developmental path for naïve T cells to differentiate into effector and memory CD8 T cells. Through the integration of antigenic, co-stimulatory and inflammatory signals, a heterogeneous population of effector CD8 T cells is created. Viruses such as lymphocytic choriomeningitis virus (LCMV), stimulate a massive expansion of antigen-specific CD8 T cells that peaks at day 8–10 post infection (PI). At the height of the expansion phase, at least two effector CD8 T-cell subset populations can be identified: the short-lived effector cells (SLECs) and memory precursor effector cells (MPECs). Following viral clearance, the majority of the SLECs are eliminated via Bim-dependent apoptosis, while the MPECs undergo further differentiation to populate the memory CD8 T-cell pool. Memory CD8 T cells, although heterogeneous in their phenotype, display a well-defined set of characteristics, including enhanced proliferative capacity, augmented re-expression of effector genes upon rechallenge and the ability to self-renew and survive long-term.

The signals and factors that determine the fate of the effector CD8 T cells and their subsequent development into memory CD8 T cells have been the focus of intensive research (reviewed in Jameson and Masopust¹). Signals triggered by antigen, co-stimulatory molecules and growth factor receptor activation act in synergy to induce cell-cycle progression in quiescent, naïve T cells. The progression through the cell cycle is tightly regulated and involves the interaction of cyclins with their cognate partners, the cyclin-dependent kinases (CDKs). The cyclin–CDK complex is, in turn, under tight regulatory control by a family of proteins called the CDK inhibitors (CDKIs). One member of the Cip/Kip family of CDKIs is p27Kip1. The expression of p27Kip1 in T cells varies with the state of their development. In mature T cells, p27Kip1 is highly expressed in naïve quiescent cells but is downregulated upon mitogenic stimulation. Downregulation of p27Kip1 appears to be obligatory for naïve T cells to enter the cell cycle and undergo T-cell receptor-driven clonal expansion, and p27Kip1 has also been implicated in promoting T-cell anergy. Interestingly, the terminally differentiated status of SLECs and their susceptibility to apoptosis is associated with higher p27Kip1 protein levels. Additionally, it has been reported that the enhanced ability of memory CD8 T cells to proliferate is due to reduced expression of p27Kip1. We have previously documented that p27Kip1 is a critical regulator of CD8 T-cell homeostasis and limits the magnitude and quality of memory CD8 T cells. This study, however, did not
elucidate whether \( p27^{kip1} \) regulated CD8 T-cell memory by T-cell-intrinsic mechanisms. This is an important issue because \( p27^{kip1} \) has been implicated in regulating the function and life span of DCs, which have a crucial role in programming the differentiation of effector and memory CD8 T cells.

The focus of the present study is to dissect the T-cell-intrinsic and -extrinsic effects of \( p27^{kip1} \) on the generation and maintenance of CD8 T-cell memory. We report the development of conditional knockout mice strains in which \( p27^{kip1} \) expression is selectively extinguished in T cells and/or non-T cells. Using these mice, we find that \( p27^{kip1} \) restricts the proliferation of antigen-specific CD8 T cells at all phases of the immune response to LCMV by T-cell-intrinsic mechanisms. Although the absence of \( p27^{kip1} \) in T cells drives enhanced proliferation, the elevated proliferation is not sufficient to alter the number of memory CD8 T cells. Interestingly, deletion of \( p27^{kip1} \) in non-T cells has no significant effect on proliferation of virus-specific CD8 T cells but markedly augments the quality and quantity of memory CD8 T cells. To our knowledge, this is the first report of a cell-cycle regulator controlling the magnitude and quality of CD8 T-cell memory through non-T-cell compartment-centric mechanisms, independent of proliferation. These findings have improved our understanding of the molecular and cellular mechanisms that govern CD8 T-cell memory, which might have implications in the development of vaccines that engender potent CD8 T-cell memory and protective immunity.

**RESULTS**

**Generation and characterization of T-cell- and non-T-cell-specific \( p27^{kip1} \) knockout mice**

To determine whether \( p27^{kip1} \) regulates CD8 T-cell responses by T-cell-intrinsic or -extrinsic mechanisms, we utilized the global \( p27^{kip1} \)-deficient mice along with mice that are conditionally deficient for \( p27^{kip1} \) in T cells or non-T cells. Derivation of \( p27^{kip1} \)-deficient mice, which carry a null allele of \( p27 \) has been described elsewhere,\(^{20}\) they are herein referred to as \( p27^{-/-} \) mice. Additionally, we utilized \( p27^{loxP} \) mice, which carry a floxed \( p27^{kip1} \) allele (\( p27^{L/+} \)) as well as \( p27^{stop} \) mice, which carry an allele (\( p27^{S/-} \)) harboring a floxed transcriptional-stop cassette inserted in the \( p27^{kip1} \) promoter region.\(^{21}\) To induce T-cell-specific deletion of \( p27^{kip1} \), we crossed CD4-Cre transgenic mice (Taconic Farms, Germantown, NY, USA) that express Cre recombinase under the control of the CD4 proximal promoter, with \( p27^{loxP} \) mice for two generations. In the resulting CD4-Cre\(^+\)/\( p27^{L/+}\)/\( p27^{S/-} \) mice, which we refer to as T-OFF mice, Cre recombinase expression will lead to deletion of the \( p27^{kip1} \) gene in thymocytes at the double-positive stage. Using a similar breeding strategy, we also created mice that lack \( p27^{kip1} \) gene expression in all the cell types, with the exception of the T-cell compartment. Specifically, we crossed CD4-Cre transgenic mice with \( p27^{stop} \) mice for two generations. The offspring from these crosses, CD4-Cre\(^+\)/\( p27^{S/-} \), are expected to express \( p27^{kip1} \), under control of the endogenous promoter, exclusively in T cells, whereas all non-T cells lack \( p27^{kip1} \) expression; we refer to these mice as T-ON mice in this manuscript. We confirmed the cell type-specific deletion of \( p27^{kip1} \) in T cells and non-T cells using western blot analysis (Figure 1a). As expected, the global \( p27^{kip1} \)-deficient \( p27^{-/-} \) mice had undetectable levels of \( p27^{kip1} \) protein in both T cells and non-T cells. T-OFF mice exhibit full ablation of \( p27^{kip1} \) protein expression in the T-cell compartment, whereas T cells from T-ON mice showed \( p27^{kip1} \) protein levels comparable with that of wild-type (WT) mice (Figure 1a). Conversely, in the non-T-cell fraction, \( p27^{kip1} \) protein was not detected in the T-ON mice, but T-OFF mice expressed WT levels of \( p27^{kip1} \) protein. Using reverse transcriptase PCR, we confirmed the deletion of \( p27^{kip1} \) at the level of mRNA in each of the strains of mice (not shown). The cell type-specific deletion of \( p27^{kip1} \) had no significant effect on the relative proportions of mature CD4 or CD8 T cells in the spleen (Figure 1b). Furthermore, the percentages of double-negative (CD4\(^-\)CD8\(^-\)), double-positive (CD4\(^+\)CD8\(^+\)) and single-positive (CD4\(^+\) or CD8\(^+\)) thymocytes were unaffected by deletion of \( p27^{kip1} \) in T cells or non-T cells (data not shown). To examine the possibility that infection with LCMV might corrupt Cre recombinase expression and alter the expression of \( p27^{kip1} \) in T cells and non-T cells, we infected groups of WT, \( p27^{-/-} \) and T-OFF mice with LCMV. At days 8 and 30 after LCMV infection, T cells and non-T cells were purified from spleens, and the expression of \( p27^{kip1} \) mRNA was quantified by real-time PCR.

![Figure 1](https://example.com/figure1.png)  
**Figure 1** Characterization of T-OFF and T-ON mice. Splenocytes from naive WT, \( p27^{-/-} \), T-OFF and T-ON mice were used to purify total T-cell and non-T-cell populations utilizing the MACS system. (a) Cellular \( p27^{kip1} \) protein levels were quantified using SDS-PAGE followed by immunoblotting with anti-\( p27^{kip1} \) antibody. Probing for \( \beta\)-actin was used as a loading control. Figure shows \( p27^{kip1} \) and \( \beta\)-Actin expression in T cells and non-T cells from two representative mice for each group. (b) Splenocytes from naive WT, \( p27^{-/-} \), T-OFF and T-ON mice were collected and stained with anti-CD4 and anti-CD8. Representative dot plots are gated on total splenocytes, and numbers represent the percentages of CD4 and CD8 positive T cells.
This analysis showed that even after LCMV infection, only non-T cells but not T cells in T-OFF mice expressed readily detectable levels of p27Kip1 mRNA (Supplementary Figure S1); the purity of T cells obtained from T-OFF mice was ~80% and therefore very low levels of p27Kip1 mRNA in this cellular fraction likely originated from contaminating non-T cells.

Loss of p27Kip1 in T cells or non-T cells does not affect accumulation of CD8 T cells during the primary CD8 T-cell response to an acute LCMV infection

To determine whether the loss of p27Kip1 in T cells and/or non-T cells affected activation and expansion of CD8 T cells, groups of WT, p27−/−, T-OFF and T-ON mice were infected with LCMV. At day 8 PI, the virus-specific CD8 T-cell responses were assessed in the spleen. At day 8 PI, the total numbers of activated (CD44hi) and naive (CD44lo) CD8 T cells were comparable in all the four groups of mice. (Figure 2a). Likewise, the percentage and the total numbers of LCMV-specific CD8 T cells in the spleen of WT, p27−/−, T-OFF and T-ON mice were not significantly different (Figure 2b). Additionally, LCMV-specific CD8 T cells from all the four groups of mice displayed the expected CD44hi/CD62Llo phenotype, and the effector CD8 T cells expressed similar levels of the cell surface receptors CD27 and CD122 (Figure 2c). Thus, data in Figure 2 suggested that ablation of p27Kip1 in T cells or non-T cells did not affect the clonal expansion, phenotype or function of effector CD8 T cells. (Figure 2).

Loss of p27Kip1 in non-T cells regulates CD8 T-cell memory

At the peak of the T-cell response, the pool of effector cells can be classified into two subsets based on the cell surface expression of interleukin (IL)-7Rα (CD127) and killer cell lectin-like receptor subfamily G member 1 (KLRG-1). The SLECs (KLRG-1lo/CD127hi) represent the more terminally differentiated cell type that have the propensity to undergo apoptosis during the contraction phase, whereas the MPECs (KLRG-1hi/CD127lo) have the potential to survive and further differentiate into long-lived memory CD8 T cells. On day 8 PI, the deletion of p27Kip1 in T cells and/or non-T cells had no significant impact on the number of SLECs or MPECs (Figure 2d).

A key feature of effector CD8 T cells is their ability to rapidly produce cytokines such as interferon γ (IFNγ) and express effector molecules like granzyme B. To examine possible alterations in the function of effector CD8 T cells, we measured IFNγ production and granzyme B levels in NP396-specific CD8 T cells from WT, p27−/−, T-OFF and T-ON mice on day 8 PI. CD8 T cells from all the groups of mice produced readily detectable IFNγ upon stimulation with the NP396 peptide (Figure 2e). The levels of IFNγ produced by CD8

Granzyme B expression in LCMV-specific CD8 T cells.

The FACS histograms are gated on NP396-specific CD8 T cells from WT, p27−/−, T-OFF and T-ON mice. The numbers on the histograms represent the mean fluorescence intensity (MFI) for the indicated protein. (d) On day 8 PI, total splenocytes were stained with DNP396 tetramer, anti-CD8, anti-CD127 and anti-KLRG-1, and the total number of SLECs (KLRG-1hi/CD127lo) and MPECs (CD127lo/KLRG-1lo) were quantified by flow cytometry. (e) IFNγ production by LCMV-specific CD8 T cells. On day 8 PI, splenocytes from WT, p27−/−, T-OFF and T-ON mice were stimulated with NP396 peptide for 5 h directly ex-vivo. Following stimulation, cells were stained for cell surface CD8 and intracellular IFNγ. Representative dot plots are gated on total splenocytes, and the numbers indicate the MFI for IFNγ at 8 days PI.

Granzyme B expression in LCMV-specific CD8 T cells. The FACS histograms are gated on NP396-specific CD8 T cells from WT, p27−/−, T-OFF and T-ON mice and show the percentages of granzyme B-positive cells. Data are from three independent experiments with 3–5 mice/group/experiment. Error bars represent the s.e.m.
T cells, as measured by the mean fluorescence intensity for IFN-\(\gamma\) staining, were similar in all the four groups of mice. Likewise the levels of granzyme B in NP396-specific effector CD8 T cells did not significantly differ between groups of mice (Figure 2e). Collectively, data in Figure 2 suggest that the loss of p27\(^{kip1}\) in T cells and/or non-T cells did not appreciably affect the clonal expansion or effector function of CD8 T cells.

**CDKI p27\(^{kip1}\) governs the contraction of effector CD8 T cells by T-cell-extrinsic mechanism(s) independent of proliferation**

Following LCMV clearance, \(\sim 90\%\) of effector CD8 T cells are eliminated between days 8 and 30 PI. To probe the T-cell-intrinsic versus T-cell-extrinsic role of p27\(^{kip1}\) in the contraction of effector CD8 T cells, we infected groups of WT, p27\(^{-/-}\), T-OFF and T-ON mice with LCMV and analyzed virus-specific CD8 T-cell responses at day 30 PI. The total numbers of LCMV-specific CD8 T cells in spleens of p27\(^{-/-}\) mice were significantly higher (\(\sim 3\)-fold) as compared with WT mice (Figure 3a). Surprisingly, a similar increase in the numbers of LCMV-specific CD8 T cells was observed in T-ON mice but not in the T-OFF mice (Figure 3a). To define the magnitude of CD8 T-cell contraction in the spleen for the four groups of mice, we calculated the fold loss for NP396 and GP33-specific CD8 T cells in the interval between days 8 and 30 PI (Figure 3b). In the WT mice, virus-specific CD8 T cells experienced a 7.3–8.5-fold contraction. Strikingly, there was a marked reduction in the contraction of LCMV-specific CD8 T cells in p27\(^{-/-}\) mice (2.7–3.1-fold). The contraction of effector CD8 T cells in T-OFF mice was slightly lower than in WT mice (3.6–7.2-fold), whereas the T-ON mice displayed considerably reduced contraction (3.2–3.9-fold). These data suggested that p27\(^{kip1}\) in non-T cells promotes contraction of effector CD8 T cells. As effector/memory CD8 T cells are found in both lymphoid and non-lymphoid organs, we also assessed whether p27\(^{kip1}\) deficiency in T cells or non-T cells affected the expansion and contraction of LCMV-specific CD8 T cells in non-lymphoid organs, such as the liver. Unlike in the spleen (Figures 2 and 3), global or conditional deficiency for p27\(^{kip1}\) in T cells or non-T cells did not significantly \((P<0.05)\) alter the number of LCMV-specific CD8 T cells in the liver at both days 8 and 30 PI (Supplementary Figure S2).

**CDKI p27\(^{kip1}\) has been shown to function as a critical negative regulator of cell-cycle entry of T cells.**

To evaluate whether increased proliferation underlies the reduced contraction of effector CD8 T cells in p27\(^{-/-}\) and T-ON mice, we measured the proliferation of LCMV-specific CD8 T cells on days 8 and 30 PI by staining for Ki-67 (Figure 3c). The percentages of Ki-67\(^{+}\) cells among virus-specific CD8 T cells were significantly increased in global p27\(^{kip1}\)-deficient mice both on day 8 and on day 30 PI, as compared with those in WT mice. The percentages of proliferating Ki-67\(^{+}\) virus-specific CD8 T cells in T-ON mice were equivalent to those in

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**Figure 3** Contraction of effector CD8 T cells is regulated by p27\(^{kip1}\) by T-cell-extrinsic mechanisms independent of proliferation. (a) At day 30 PI, splenocytes from WT, p27\(^{-/-}\), T-OFF and T-ON mice were stained with anti-CD8 and D\(^{b}\) tetramers. Bar graph shows the total number of D\(^{b}\)/NP396- (left) and D\(^{b}\)/GP33- (right) specific CD8 T cells. (b) Fold contraction of LCMV-specific CD8 T cells between days 8 and 30 PI. To illustrate the difference in contraction kinetics between WT, p27\(^{-/-}\), T-OFF and T-ON mice, the fold differences in the total numbers of virus-specific CD8 T cells between day 8 (from Figure 2) and day 30 were calculated for each group. (c) At days 8 and 30 PI, splenocytes from WT, p27\(^{-/-}\), T-OFF and T-ON mice were stained with anti-CD8, D\(^{b}\)/NP396 tetramer and anti-Ki67; the percentages of Ki-67 positive NP396-specific cells are shown for day 8 (left) and day 30 PI (right). (d) NP396-specific SLECs (KLRC1\(^{-/-}\)/CD127\(^{hi}\)) and MPECs (CD127\(^{hi}\)/KLRC1\(^{lo}\)) at day 30 PI. Bar graph shows the total number of SLECs and MPECs in spleens of WT, p27\(^{-/-}\), T-OFF and T-ON mice. (e) As a measure of contraction, we calculated the percentages of SLECs and MPECs from day 8 PI that survived until day 30 PI. (f) The percentage of Ki67\(^{+}\) cells among SLEC and MPEC subsets of NP396-specific CD8 T cells was determined by flow cytometry at day 30 PI. Data is expressed as the percentage of Ki-67-positive cells among NP396-specific CD8 T-cell effector subsets. Data are from three independent experiments with 3–5 mice/group/experiment. Error bars represent the s.e.m. and asterisk indicates statistical significance at \(P<0.05\).
WT mice. Interestingly, in the T-OFF mice, proliferation of virus-specific CD8 T cells was substantially augmented, to an even greater degree than those of p27<sup>−/−</sup> mice (day 8: 50% and day 30: 300% increase over WT). Thus, contraction of effector CD8 T cells was substantially reduced in T-ON mice, without detectable alterations in cellular proliferation. The finding that increased proliferation minimally affected the expansion and contraction of LCMV-specific CD8 T cells in T-OFF mice raised the possibility that enhanced apoptosis might have offset the effects of proliferation. To examine this possibility, we quantified the percentages of Annexin V<sup>+</sup> LCMV-specific CD8 T cells in the spleen of WT and T-OFF mice at days 8 and 30 PI. At day 8 PI, the percentages of Annexin V<sup>+</sup> LCMV-specific CD8 T cells in T-OFF mice were slightly lower but not significantly different (P < 0.05) compared with those in WT mice (Supplementary Figure S3). In parallel studies, we also quantified the levels of Bim and Bcl-2 in LCMV-specific CD8 T cells from WT and T-OFF mice. The quantified mean fluorescence intensities for Bim and Bcl-2 were used to calculate the Bim:Bcl-2 ratios (Supplementary Figure S3). At day 8 PI, the Bim:Bcl-2 ratios were significantly (P < 0.05) higher in LCMV-specific CD8 T cells from T-OFF mice, as compared with those in WT mice. Although we did not detect significant differences in percentages of Annexin V<sup>+</sup> cells between CD8 T cells from WT and T-OFF mice, it is possible that the increased Bim:Bcl-2 ratio might have increased the susceptibility of T-OFF CD8 T cells to apoptosis during the contraction phase. Collectively, data presented in Figure 3 illustrated that p27<sup>Kip1</sup> deficiency: (1) constrained the proliferation of virus-specific CD8 T cells by T-cell-intrinsic mechanisms; (2) promoted contraction of effector CD8 T cells through non-T cells by mechanism(s) that does not include proliferation.

Next, we quantified the numbers of SLECs and MPECs in spleens of WT, p27<sup>−/−</sup>, T-OFF and T-ON mice at day 30 PI. The numbers of both SLECs and MPECs in the spleens of p27<sup>−/−</sup> and T-ON mice were significantly higher than in WT or T-OFF mice (Figure 3d). Based on the numbers of SLECs and MPECs present at days 8 (Figure 2d) and 30 PI (Figure 3d), we calculated the magnitude of contraction of these subsets for different groups of mice (Figure 3e). Figure 3e shows that SLECs contracted markedly in all the groups of mice; 88–93% of the SLECs were lost between days 8 and 30 PI. Although 40% of the MPECs were lost between days 8 and 30 PI in WT mice, the number of MPECs increased substantially in both p27<sup>−/−</sup> and T-ON mice in the same interval (Figure 3e). To understand the underlying mechanism, we assessed the proliferation of SLEC and MPEC subsets in all four groups of mice at day 30 PI (Figure 3f). Regardless of their differentiation status (SLECs or MPECs), global proliferation of SLECs and MPECs in T-ON mice was significantly higher than in WT mice. As another index of the quality of memory CD8 T cells, we assessed whether p27<sup>Kip1</sup> deficiency augmented the proliferative renewal of memory CD8 T cells by T-cell-intrinsic mechanisms. We assessed the cell-cycle status of LCMV-specific memory CD8 T cells by staining for Ki-67. Data in Figure 4b showed that the percentages of proliferating Ki-67<sup>+</sup> memory CD8 T cells were significantly higher in p27<sup>−/−</sup> and T-ON mice as compared with WT and T-OFF mice; the percentages of Ki-67<sup>+</sup> cells in WT and T-ON mice were not statistically different (P < 0.05). These data suggested that the loss of p27<sup>Kip1</sup> in T cells enhanced the proliferative renewal of memory CD8 T cells. Based on these findings, we infer that p27<sup>Kip1</sup> limited the proliferative renewal of LCMV-specific memory CD8 T cells by T-cell-intrinsic mechanisms.

Memory CD8 T cells are maintained at relatively stable levels by proliferative renewal driven by homeostatic cytokines, including IL-7 and IL-15.32 It has been previously reported that p27<sup>Kip1</sup>-deficient T cells exhibit hyper-proliferative responses to cytokines.23,24 Therefore, it was of interest to determine whether p27<sup>Kip1</sup> regulated the proliferative renewal of memory CD8 T cells by T-cell-intrinsic mechanism(s). We assessed the cell-cycle status of LCMV-specific memory CD8 T cells by staining for Ki-67. Data in Figure 4b showed that the percentages of proliferating Ki-67<sup>+</sup> memory CD8 T cells were significantly higher in p27<sup>−/−</sup> and T-ON mice as compared with WT and T-OFF mice; the percentages of Ki-67<sup>+</sup> cells in WT and T-ON mice were not statistically different (P < 0.05). These data suggested that the loss of p27<sup>Kip1</sup> in T cells enhanced the proliferative renewal of memory CD8 T cells. Based on these findings, we infer that p27<sup>Kip1</sup> limited the proliferative renewal of LCMV-specific memory CD8 T cells by T-cell-intrinsic mechanisms.

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We examined whether loss of p27<sup>Kip1</sup> in T cells and/or non-T cells affected the expression of CD27, a molecule implicated in determining the protective efficacy of memory CD8 T cells.25,26 Memory CD8 T cells in all the groups of mice were uniformly CD27<sup>+</sup> and therefore p27<sup>Kip1</sup> might not regulate CD27 expression (Figure 4c). We also assessed whether p27<sup>Kip1</sup> controlled the differentiation of central CD62L<sup>+</sup> and effector CD62L<sup>+</sup> memory CD8 T cells. Figure 4d shows that deletion of p27<sup>Kip1</sup> in T cells and/or non-T cells did not affect the relative proportions of effector or central memory CD8 T-cell populations.

As another index of the quality of memory CD8 T cells, we assessed the ability of LCMV-specific CD8 T cells to produce cytokines, including IFN<sub>γ</sub>, TNFα (tumor necrosis factor α) and IL-2 in response to antigenic stimulation.27 Figure 4e shows that the percentages of IFN<sub>γ</sub>-producing CD8 T cells were significantly increased in the spleens of p27<sup>−/−</sup> and T-ON mice, as compared with WT and T-OFF mice. Remarkably, we observed an approximated 500% increase in the total number of IFN<sub>γ</sub>-producing LCMV-specific CD8 T cells in p27<sup>−/−</sup> and T-ON mice, as compared with WT mice (Figure 4e). More dramatic differences were evident when triple-cytokine-producing LCMV-specific CD8 T cells were compared...
between groups of mice. Not only were the percentages of triple-cytokine-producing CD8 T cells significantly higher in p27\(^{-/-}\) and T-ON mice, there was an \(\sim 10\)-fold increase in the total number of triple-cytokine-producing CD8 T cells in p27\(^{-/-}\) and T-ON mice (Figure 4f). Triple-cytokine-producing CD8 T-cell numbers in T-Off mice were comparable with those of WT mice, demonstrating that a T-cell-extrinsic mechanism might have driven the increase in multi-cytokine-producing memory CD8 T cells.

The analysis of the kinetics of IFN\(\gamma\) and triple-cytokine-producing LCMV-specific CD8 T-cell responses from day 8 through day 90 PI clearly demonstrates the impact p27\(^{kip1}\) has on the population size of triple-cytokine-producing, virus-specific CD8 T cells following an acute LCMV infection (Figure 4g). The absence of p27\(^{kip1}\) in non-T cells lead to a preferential enrichment of triple-cytokine-producing memory CD8 T cells between day 8 and day 30 PI, and this accumulation was sustained long-term. Data in Figure 4g also clearly demonstrates that the deletion of p27\(^{kip1}\) in T cells alone is not sufficient to confer this phenotype but is dependent on the deletion of p27\(^{kip1}\) in non-T cells.

**Priming of memory CD8 T cells by p27\(^{kip1}\)-deficient DCs**

Data presented in Figure 4 demonstrated that the deletion of p27\(^{kip1}\) in the non-T-cell compartment enhanced the quality and quantity of memory CD8 T cells. Because DCs are known to program differentiation of effector and memory T cells during an acute viral infection,\(^1\) we decided to investigate DCs as a non-T-cell population that are potentially involved in enhancing CD8 T-cell memory in p27\(^{-/-}\) and T-ON mice. To test whether p27\(^{kip1}\) deficiency in the DCs alters differentiation of memory CD8 T cells, we derived DCs by stimulating bone marrow cells of WT and p27\(^{-/-}\) mice with FLT3 (FMS-like tyrosine kinase 3) ligand. Subsequently, FLT3 ligand-induced DCs were induced to undergo maturation by stimulating with lipopolysaccharide. Consistent with mature DCs, lipopolysaccharide-stimulated WT CD11c\(^{+}\) DCs display...
increased levels of MHC II (major histocompatibility complex class II) and co-stimulatory molecules CD80, CD86 and CD40, as compared with unstimulated DCs (Figure 5a and data not shown). CD11c\(^+\) DCs, derived from p27\(^{−/−}\)/C0 mice, displayed a higher expression of MHC II and costimulatory molecules, as compared with WT DCs (Figure 5a).

To test whether intrinsic differences in DCs between WT and p27\(^{−/−}\)/C0 mice underlie altered memory CD8 T-cell priming in vivo, we immunized WT mice with LCMV GP33 peptide-pulsed mature bone marrow-derived DCs from WT and p27\(^{−/−}\)/C0 mice. At days 8 and 21 after DC immunization, we quantified the number of GP33-specific CD8 T cells by intracellular cytokine staining (Figure 5b). The numbers of IFN\(\gamma\)-producing GP33-specific CD8 T cells in p27\(^{−/−}\)/C0 DC-immunized mice were comparable with those in WT DC-immunized mice. Additionally, the percentages of triple-cytokine-producing GP33-specific CD8 T cells in p27\(^{−/−}\)/C0 DC-immunized mice were comparable with those in WT DC-immunized mice. Additionally, the percentages of triple-cytokine-producing GP33-specific CD8 T cells in p27\(^{−/−}\)/C0 DC-immunized mice were comparable with those in WT DC-immunized mice. The percentages of SLECs and MPECs among GP33-specific CD8 T cells were comparable in mice immunized with WT and p27\(^{−/−}\)/C0 DCs (data not shown). Further, we compared the ability of WT and p27\(^{−/−}\)/C0 DCs to persist after adoptive transfer into WT mice. For at least until 72 h after transfer, we did not find significant differences in the numbers of WT and p27\(^{−/−}\)/C0 DCs in the spleen of WT-recipient mice (data not shown). Taken together, data in Figure 5 failed to demonstrate that bone marrow-derived FLT3 ligand-induced p27\(^{Kip1}\)-deficient DCs have an enhanced ability to prime CD8 T-cell memory.

**DISCUSSION**

Induction of immunological memory is the basis of vaccinations, and understanding the molecular and cellular basis of B- and T-cell memory is vital for development of effective vaccines against diseases such as AIDS, tuberculosis and malaria.\(^{28}\) Therefore, there is strong impetus to decipher the mechanisms that regulate the establishment and maintenance of durable T-cell memory.\(^1\) Protective immunity depends upon the number and functional quality of memory CD8 T cells, but the underlying mechanisms that govern these two attributes of CD8 T-cell memory are not well understood. We have previously shown that the CDKI p27\(^{Kip1}\) is a critical negative regulator of the magnitude and quality of memory CD8 T cells.\(^{16}\) In this study, we confirm these results and further investigate whether regulation of CD8 T-cell memory by p27\(^{Kip1}\) occurs by T-cell-intrinsic or T-cell-extrinsic mechanisms. By conditional ablation of p27\(^{Kip1}\) in T cells and non-T cells, we show that p27\(^{Kip1}\) functions as an integral brake of the proliferation of antigen-specific CD8 T cells during expansion, contraction and memory phase of the CD8 T-cell response, by T-cell-intrinsic mechanisms. However, the most intriguing finding from this study is that p27\(^{Kip1}\) activity in non-T cells effectively limits the number of high-quality polyclonally-producing memory CD8 T cells.

![Figure 5](image-url)
and that this limitation results from mechanisms independent of T-cell proliferation. These findings have implications in targeting p27\(^{kip1}\) activity in non-T cells to enhance vaccine-induced CD8 T-cell memory.

Consistent with the established role for p27\(^{kip1}\) as a negative regulator of cellular proliferation, we find that global deficiency for p27\(^{kip1}\) or loss of p27\(^{kip1}\) exclusively in T cells results in enhanced proliferation of LCMV-specific CD8 T cells during all phases of the CD8 T-cell response: expansion, contraction, and memory. These findings suggest that p27\(^{kip1}\) regulates CD8 T-cell proliferation by T-cell-intrinsic mechanisms. However, it is indeed unexpected and intriguing that increased proliferation of p27\(^{kip1}\)-deficient CD8 T cells fails to induce a net increase in the number of CD8 T cells in p27\(^{−/−}\) or T-_OFF mice during clonal expansion. It has been reported that the balance of Bim (pro-apoptotic) and Bcl-2 (anti-apoptotic) might control the survival of effector and memory CD8 T cells.\(^{28,30}\) Consistent with this idea, we find that the Bim:Bcl-2 ratios in effector CD8 T cells from T-Off mice were higher than in effector cells from WT mice. Therefore, it is possible that the increased rate of proliferation of p27\(^{kip1}\)-deficient CD8 T cells in T-Off mice might be offset by concurrent FOXP3-induced Bim-dependent apoptosis.\(^{31}\)

How did ablation of p27\(^{kip1}\) in non-T cells increase the number of memory CD8 T cells? The abundance of memory CD8 T cells induced during an immune response is related to the number of MPECs induced during the primary CD8 T-cell response.\(^{28,32}\) At the peak of the CD8 T-cell response to LCMV (day 8 PI), the numbers of MPECs in the spleens of T-ON and p27\(^{−/−}\) mice are similar to those in WT and T-Off mice. However, at day 30 PI, the numbers of MPECs in p27\(^{−/−}\) and T-ON mice are greater than in WT or T-Off mice. Thus, diminished contraction and/or enhanced accumulation of MPECs likely underlies the increase in the number of memory CD8 T cells in T-ON and p27\(^{−/−}\) mice. The slight reduction in the contraction of MPECs in T-Off mice might be related to increased proliferation of MPECs (Figures 3b and f), which in turn modestly elevated the number of memory CD8 T cells (Figure 4g). In the T-ON mice, the increase in the number of memory CD8 T cells can be linked to a reduction in contraction and/or increased accumulation of MPECs, which cannot be explained by augmented proliferation. Therefore, we propose that p27\(^{kip1}\) activity in non-T cells inhibits the survival and/or accumulation of MPECs in LCMV-infected mice. Note that the number of MPECs in p27\(^{−/−}\) mice is higher than in T-ON and WT mice at day 30 PI. It is possible that in the global p27\(^{kip1}\)-deficient mice, increased proliferation (induced by T-cell-intrinsic loss of p27\(^{kip1}\)) along with loss of pro-apoptotic effects (dependent upon p27\(^{kip1}\) activity in non-T cells) during contraction additively inflate the number of memory CD8 T cells. At day 8 PI, a proportion of LCMV-specific CD8 T cells in all the groups of mice were KLRG-1\(^{−/−}\)/CD127\(^{−/−}\) (not shown), which are considered as early effectors. Therefore, it is also possible that more of these early effectors could have differentiated into MPECs between days 8 and 30 PI in p27\(^{−/−}\) and T-ON mice but not in WT or T-Off mice. Cytokine production by memory CD8 T cells, in particular autocrine IL-2 production, has been shown to be critical for the expansion of memory CD8 T responses during a secondary response.\(^{33}\) Here, we report that global p27\(^{kip1}\) deficiency or loss of p27\(^{kip1}\) in non-T cells markedly increases the abundance of the triple-cytokine- (IL-2 in particular) producing memory CD8 T cells. Again, p27\(^{kip1}\) activity in non-T cells appears to promote the contraction of triple-cytokine-producing CD8 T cells in WT and T-Off mice (Figure 4g).

How does p27\(^{kip1}\) regulate CD8 T-cell memory via non-T cells? A popular candidate cell that can modulate the development of CD8 T-cell memory is the DC.\(^{19}\) The constellation of signals delivered by the DCs to naive T cells at the time of activation initiates a program of differentiation that guides the formation of effector and memory CD8 T cells.\(^{34}\) Does p27\(^{kip1}\) deficiency enhance the DCs’ ability to prime memory CD8 T cells? We find that in vitro FLT3-induced bone marrow-derived p27\(^{kip1}\)-deficient DCs are not significantly better than WT DCs in inducing polyclonal polyclonal cytokine-producing memory CD8 T cells in vivo. Based on this result, we infer that in vitro-derived p27\(^{kip1}\)-deficient DCs may not possess greater intrinsic ability to prime larger numbers of polyclonal cytokine-producing memory CD8 T cells, as compared with WT DCs. FLT3-induced DCs are believed to mimic the functional attributes of CD8\(^{+}\)/CD4\(^{−}\) DCs, which are known to have a key role in priming CD8 T-cell responses in vivo.\(^{35,36}\) Although our results suggest that p27\(^{kip1}\) deficiency might not enhance the intrinsic ability of FLT3-induced DCs to prime memory CD8 T cells, we cannot formally exclude the possibility that the properties of other subsets of p27\(^{kip1}\)-deficient DCs present in p27\(^{−/−}\) and T-ON mice might enhance the number of memory CD8 T cells. It should be noted that, there is increasing evidence that stromal cells in the secondary lymphoid organs modulate immunological memory (reviewed in Tokoyoda et al.\(^{37,38}\)). Therefore, the possibility exists that p27\(^{kip1}\) activity in stromal cells controls the differentiation of effector and memory CD8 T cells in lymphoid tissues.\(^{39}\) It would be enlightening to examine if and how p27\(^{kip1}\) controls CD8 T-cell memory by modulating the homeostasis of stromal cells in lymphoid tissues.

A thorough understanding of the cellular and molecular mechanisms that govern the magnitude and quality of CD8 T-cell memory is crucial for the development of effective vaccines that engender durable immunity against intracellular pathogens and cancer. In the present study, we ascribe a novel role for p27\(^{kip1}\) that is independent of its CDKI activity in regulating the number and quality of memory CD8 T cells. Here, we delineate the T-cell-intrinsic, anti-proliferative activity of p27\(^{kip1}\) from its role as a factor that suppresses the development of CD8 T-cell memory through non-T cells. These studies suggest that targeting p27\(^{kip1}\) activity in non-T cells might be a strategy to enhance vaccine-induced CD8 T-cell memory.

**METHODS**

**Mice and viral infection**

Derivation of p27\(^{−/−}\) mice and mice carrying either the floxed p27\(^{kip1}\) alleles (p27\(^{loxP}\)) or a floxed Neomycin cassette inserted in the p27\(^{kip1}\) allele (p27\(^{STOP}\)) have been described elsewhere.\(^{21}\) To induce T-cell-specific deletion of p27\(^{kip1}\), we crossed p27\(^{loxP}\) mice with the CD4-Cre transgenic mice (Taconic Farms) that express Cre recombinase under the control of the CD4 proximal promoter to generate the T-Off mice. To create T-ON mice that lack the p27\(^{kip1}\) gene in all cell types with the exception of the T-cell compartment, we crossed p27\(^{STOP}\) mice with the CD4-Cre transgenic mice. Litterate WT mice were used as controls. Mice were infected intraperitoneally with 2 \(×\) 10\(^{6}\) PFU (plaque-forming units) of LCMV-Armstrong to induce an acute infection. Infectious LCMV was quantified by a plaque assay on Vero cells, as described previously.\(^{23}\) For experiments involving derivation and transfer of bone marrow-derived DCs, WT and p27\(^{−/−}\) mice on the C57BL/6 background were used. Mice used in experiments were between the ages of 6–8 weeks, and all experiments were performed in accordance with the protocols approved by the University of Wisconsin School of Veterinary Medicine Institutional Animal Care and Use Committee (IACUC). The animal committee mandates that institutions and individuals using animals for research, teaching and/or testing much acknowledge and accept both legal and ethical responsibility for the animals under their care, as specified in the Animal Welfare Act (AWA), the associated Animal Welfare Regulations (AWRs), the Declaration of Helsinki and Public Health Service (PHS) Policy.

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Quantitative reverse transcriptase PCR

T cells and non-T cells were purified from spleens of WT, p27–/–, T-ON mice using the anti-CD90.2 MACS cell separation system (Miltenyi Biotec, Auburn, CA, USA). Purity of cells was confirmed to be 80–90% by flow cytometry. Total RNA was extracted from the purified cells using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Quantitative real-time PCR was performed using POWERSYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) and data were normalized using 18S rRNA values. Applied Biosystems 7300 Real-Time PCR System was used for this analysis.

Western blot analysis

T cells and non-T cells were purified from the spleens as above. Cells were subsequently lysed in RIPA buffer (50 mM Tris, 150 mM NaCl, 2 mM EDTA, 10 mM 0.1% SDS, 1% Triton X-100) and total protein levels in each lysate were determined by the Bicinchoninic Acid protein assay (Pierce, Rockford, IL, USA). Samples containing 15 μg of protein were resolved on a 12% SDS-PAGE. The p27kip1 protein in each sample was detected using a mouse primary antibody specific for p27kip1 (BD Bioscience, San Jose, CA, USA), followed by a sheep anti-mouse IgG HRP-conjugated secondary antibody (GE Healthcare, Buckinghamshire, UK). Bands were visualized using chemiluminescence reagents (THERMO Fisher, Rockford, IL, USA). Blots initially probed for p27kip1 were subsequently stripped and re-probed to detect β-Actin (Sigma-Aldrich, St Louis, MO, USA) to serve as a loading control.

Flow cytometry

Single-cell suspensions of cells from the spleen, liver or peripheral blood were prepared as previously described.2 Mononuclear cells were stained with D6 MH C class I tetramers, specific for the LCMV epitope NP396–404 (NP396) and GP33–41 (GP33). In some experiments, cells were co-stained with anti-CD44, anti-LFA-1, anti-CD62L, anti-CD27, anti-CD122, anti-CD27 and anti-CD127 and anti-KLRG-1 antibodies. All antibodies were purchased from BD Biosciences; e Bioscience (San Diego, CA, USA) or Southern Biotech (Birmingham, AL, USA). Cells were fixed in 2% paraformaldehyde and analyzed with FACS Calibur or LSR II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). For intracellular cytokine staining, splenocytes were stimulated, cells were stained for surface CD8 and intracellular IFNγ, TNFα and IL-2 using a Cytofix/Cytoperm intracellular staining kit (BD Biosciences). Granzyme B, Ki-67, Bim, Bcl-2 and Annexin V stainings were performed as previously described.31

Derivation and transfer of bone marrow-derived DCs

Bone marrow derived DCs were generated as previously described.40 Briefly, bone marrow cells from WT and p27–/– mice were cultured in 10% FCS containing 100 ng/ml mouse FLT3L (Peprotec, Rocky Hill, NJ, USA) for 9 days. Lipopolysaccharide (500 ng/ml–1, Sigma-Aldrich, St Louis, MO, USA) was then added for 24 h to induce maturation. Maturation was assessed via flow cytometry and cells were pulsed with 2 μm of GP33 peptide for 2 h. Cells were washed extensively, and 5 × 106 CD11c+ve mature peptide-pulsed DCs were administered to WT C57BL/6 mice by intravenous injection.

Statistical analysis

Where indicated, P values were determined by the two-tailed Student’s t-test, and significance was defined at P<0.05.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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