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T Cell-Intrinsic and Extrinsic Mechanisms of p27^{Kip1} in the Regulation of CD8 T Cell Memory

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Abstract

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 p27^{Kip1} functions as a negative regulator of the cell cycle in various cell types including T cells and it has been implicated in regulating cellular processes including differentiation, transcription and migration. Here, we investigated whether p27^{Kip1} regulates CD8 T cell memory by T cell-intrinsic or T cell-extrinsic mechanisms, by conditional ablation of p27^{Kip1} in T cells or non-T cells. Studies of T cell responses to an acute viral infection show that p27^{Kip1} 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 p27^{Kip1} deficiency minimally affects the primary expansion or the magnitude of CD8 T cell memory. Unexpectedly, p27^{Kip1} 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 p27^{Kip1} deficiency in immunizing DCs fail to enhance CD8 T cell memory. Nevertheless, we have delineated the T cell-intrinsic, anti-proliferative activities of p27^{Kip1} 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.

Keywords

CD8 T cells; Cell cycle; Memory; p27^{Kip1}; Proliferation

INTRODUCTION

Encounter with mature, antigen-loaded dendritic cells 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

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population of effector CD8 T cells is created [1]. 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) [2]. 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) [3]. Following viral clearance, the majority of the SLECs are eliminated via Bim-dependent apoptosis [4, 5], 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 [6].

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 [1]). 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 [7]. The progression through the cell cycle is tightly regulated and involves the interaction of cyclins with their cognate partners, the cyclin-dependent kinases (CDK) [8]. The cyclin-CDK complex is in turn under tight regulatory control by a family of proteins called the CDK inhibitors (CDKI) [9]. One member of the Cip/Kip family of CDKI is $p27^{Kip1}$. The expression of $p27^{Kip1}$ in T cells varies with the state of their development. In mature T cells, $p27^{Kip1}$ is highly expressed in naïve quiescent cells, but is downregulated upon mitogenic stimulation [10–12]. Downregulation of $p27^{Kip1}$ appears to be obligatory for naïve T cells to enter the cell cycle and undergo TCR-driven clonal expansion, and $p27^{Kip1}$ has also been implicated in promoting T cell anergy [13, 14]. Interestingly, the terminally differentiated status of SLECs and their susceptibility to apoptosis is associated with higher $p27^{Kip1}$ protein levels[15]. Additionally, it has been reported that the enhanced ability of memory CD8 T cells to proliferate is due to reduced expression of $p27^{Kip1}$ [12]. We have previously documented that $p27^{Kip1}$ is a critical regulator of CD8 T cell homeostasis and limits the magnitude and quality of memory CD8 T cells [16]. 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 dendritic cells [17, 18], which play a crucial role in programming the differentiation of effector and memory CD8 T cells [19].

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 p27loxP mice, which carry a floxed $p27^{Kip1}$ allele ($p27^{L+}$) as well as p27stop 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) that express Cre recombinase under the control of the CD4 proximal promoter, with p27loxP mice for two generations. In the resulting CD4-Cre^{+}/$p27^{L+/L+}$ 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 cell types with the exception of the T cell compartment. Specifically, we crossed CD4-Cre transgenic mice with p27stop mice for two generations. The offspring from these crosses, CD4-Cre^{+}/$p27^{S-/S-}$, 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 to 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 RT-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. 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 $p27^{Kip1}$ mRNA (Supplemental Figure 1); the purity of T cells obtained from T-OFF mice was ~80% and therefore very low levels of $p27^{Kip1}$ mRNA in this cellular fraction likely originated from contaminating non-T cells.

Loss of p27^{Kip1} 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 p27^{Kip1} 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 postinfection (PI), the virus-specific CD8 T cell responses were assessed in the spleen. At day 8 PI, the total numbers of activated (CD44^{Hi}) and naïve (CD44^{Lo}) CD8 T cells were comparable in all 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 four groups of mice displayed the expected CD44^{Hi}/CD62^{Lo} 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 p27^{Kip1} in T cells or non-T cells did not affect the activation and clonal expansion of CD8 T cells following an acute viral infection. Consistent with strong activation of CD8 T cells, LCMV was effectively controlled in all groups of mice and infectious LCMV in various tissues was below the level of detection at day 8 PI (not shown).

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 IL-7Rα (CD127) and KLRG-1. The SLECs (KLRG-1^{Hi}/CD127^{Lo}) represent the more terminally differentiated cell type that have the propensity to undergo apoptosis during the contraction phase [3], whereas the MPECs (KLRG-1^{Lo}/CD127^{Hi}) have the potential to survive and further differentiate into long-lived memory CD8 T cells [3]. On day 8 PI, the deletion of p27^{Kip1} 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 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 groups of mice produced readily detectable IFNγ upon stimulation with the NP396 peptide (Figure 2E). The levels of IFNγ produced by CD8 T cells, as measured by the Mean Fluorescence Intensity (MFI) for IFNγ staining, were similar in all 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.

CDK inhibitor p27^{Kip1} governs the contraction of effector CD8 T cells by T cell-extrinsic mechanism(s) independent of proliferation

Following LCMV clearance, ~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 (~3-fold) as compared to 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 (5.6–7.2-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. Since 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 (Supplemental Figure 2).

CDKI $p27^{Kip1}$ has been shown to function as a critical negative regulator of cell cycle entry of T cells [10, 11, 14]. To evaluate if 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 day 8 and day 30 PI by staining for Ki-67 (Figure 3C). The percentages of Ki-67^{+ve} cells amongst virus-specific CD8 T cells were significantly increased in global $p27^{Kip1}$-deficient mice both on day 8 and day 30 PI, as compared to those in WT mice. The percentages of proliferating Ki-67^{+ve} virus-specific CD8 T cells in T-ON mice were equivalent to those in 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−/− 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^{+ve} LCMV-specific CD8 T cells in spleen of WT and T-OFF mice at days 8 and 30 PI. At day 8 PI, the percentages of Annexin V^{+ve} LCMV-specific CD8 T cells in T-OFF mice were slightly lower but not significantly different ($P<0.05$) compared to those in WT mice (Supplemental Figure 3). 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 MFIs for Bim and Bcl-2 were used to calculate the Bim: Bcl-2 ratios (Supplemental Figure 3). 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 to those in WT mice. Although we did not detect significant differences in percentages of Annexin V^{+ve} 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 $p27^{Kip1}$: (1) constrained the proliferation of virus-specific CD8 T cells by T cell-intrinsic mechanism(s); (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−/−, T-OFF, and T-ON mice at day 30 PI. The numbers of both SLECs and MPECs in spleens of p27−/− 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 groups of mice; 88–93% of the SLECs were lost between days 8 to 30 PI. While 40% of the MPECs were lost between days 8 and 30 PI in WT mice, the number of MPECs increased substantially in both p27−/− 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 (p27−/−) or T cell-specific (T-OFF) deficiency for $p27^{Kip1}$ enhanced the proliferation of LCMV-specific CD8 T cells. Loss of $p27^{Kip1}$ in non-T cells of T-ON mice had a minimal impact on the proliferation of SLECs and MPECs. These findings confirmed that the greater numbers of LCMV-specific CD8 T cells (and MPECs) in T-ON mice (Figures 3A and 3D) could not be explained by enhanced proliferation of SLECs or MPECs. By extension, we propose that $p27^{Kip1}$ in non-T cells (via T cell-extrinsic effects) downregulated the accumulation of MPECs following an acute LCMV infection by mechanisms independent of proliferation.

Ablation of $p27^{Kip1}$ in non-T cells improves the quantity and quality of memory CD8 T cells whereas loss of $p27^{Kip1}$ in T cells enhances proliferative renewal of memory CD8 T cells

The quantity and quality of memory CD8 T cells determines the effectiveness of protective secondary responses. We have previously reported that global $p27^{Kip1}$ deficiency enhanced the magnitude and quality of memory CD8 T cells [16]. Here, we explored whether $p27^{Kip1}$ constrained CD8 T cell memory by T cell-intrinsic mechanism(s). Groups of WT, p27−/−, T-OFF and T-ON mice were infected with LCMV, and virus-specific CD8 T cell responses were quantified at day 90 PI. Global deletion of $p27^{Kip1}$ in p27−/− mice significantly enhanced the total numbers of LCMV-specific memory CD8 T cells (Figure 4A). Strikingly, this increase in the numbers of memory CD8 T cells was also observed in the T-ON but not in the T-OFF mice. The increase in the number of memory CD8 T cells in p27−/− and T-ON mice was at least in part attributable to larger spleen size. In summary, data in Figure 4 suggested that loss of $p27^{Kip1}$ in T cells minimally affected the quantity of CD8 T cell memory. Thus, unexpectedly, $p27^{Kip1}$ ablation solely in non-T cells was sufficient to significantly augment the number of bonafide memory CD8 T cells.

Memory CD8 T cells are maintained at relatively stable levels by proliferative renewal driven by homeostatic cytokines including IL-7 and IL-15 [22]. It has been previously reported that $p27^{Kip1}$-deficient T cells exhibit hyper-proliferative responses to cytokines [23, 24]. Therefore, it was of interest to determine whether $p27^{Kip1}$ 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^{+ve} memory CD8 T cells were significantly higher in p27−/− and T-OFF mice as compared to WT and T-ON mice; the

percentages of Ki-67^{+ve} cells in WT and T-ON mice were not statistically different ($P<0.05$). These data suggested that the loss of p27^{Kip1} in T cells enhanced the proliferative renewal of memory CD8 T cells. Based on these findings, we infer that p27^{Kip1} limited the proliferative renewal of LCMV-specific memory CD8 T cells by T cell-intrinsic mechanisms.

Next, we investigated whether increased proliferative renewal of p27^{Kip1}-deficient CD8 T cells was linked to increased expression of the IL-7 and IL-15 (CD122) receptors. The levels of CD127 and CD122 on LCMV-specific memory CD8 T cells in p27−/−, T-OFF and T-ON mice were comparable to those in WT mice (Figure 4C). Thus, augmented proliferation of memory CD8 T cells in p27^{Kip1}-deficient CD8 T cells was not linked to altered expression of IL-7/IL-15 receptors. Instead, it is likely related to enhanced intrinsic responsiveness of p27^{Kip1}-deficient memory CD8 T cells to cytokine signaling [24].

We examined whether loss of p27^{Kip1} 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 groups of mice were uniformly CD27^{Hi} and therefore p27^{Kip1} might not regulate CD27 expression (Figure 4C). We also assessed whether p27^{Kip1} controlled the differentiation of central (CD62L^{Hi}) and effector (CD62L^{Lo}) memory CD8 T cells. Figure 4D shows that deletion of p27^{Kip1} 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 IFNγ, TNFα and IL-2 in response to antigenic stimulation [27]. Figure 4E shows that the percentages of IFNγ-producing CD8 T cells were significantly increased in the spleens of p27−/− mice and T-ON mice, as compared to WT and T-OFF mice. Remarkably, we observed an approximated 500% increase in the total number of IFNγ-producing LCMV-specific CD8 T cells in p27−/− and T-ON mice, as compared to 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 ~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 to 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 IFNγ 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 dendritic cells

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 dendritic cells are known to program differentiation of effector and memory T cells during an acute viral infection[19], 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 ligand. Subsequently, FLT3 ligand-induced DCs were induced to undergo maturation by stimulating with LPS. Consistent with mature DCs, LPS-stimulated WT CD11c^{+ve} DCs display increased levels of MHC II and co-stimulatory molecules CD80, CD86 and CD40, as compared to un-stimulated DCs (Figure 5A and data not shown). CD11c^{+} DCs, derived from p27−/− mice, displayed a higher expression of MHC II and costimulatory molecules, as compared to WT DCs (Figure 5A).

To test whether intrinsic differences in DCs between WT and p27−/− 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−/− 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 IFNγ-producing GP33-specific CD8 T cells in p27−/− DC-immunized mice were comparable to those in WT DC-immunized mice. Additionally, the percentages of triple cytokine-producing GP33-specific CD8 T cells in p27−/− DC- and WT DC-immunized mice were similar. The percentages of SLECs and MPECs among GP33-specific CD8 T cells were comparable in mice immunized with WT and p27−/− DCs (data not shown). Further, we compared the ability of WT and p27−/− DCs to persist after adoptive transfer into WT mice. For at least until 72 hours after transfer, we did not find significant differences in the numbers of WT and p27−/− 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 polycytokine-producing memory CD8 T cells, 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[29, 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 FOXO3-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 [32]. At the peak of the CD8 T cell response to LCMV (day 8 PI), the numbers of MPECs in 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 (Figure 3B and 3F), 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 groups of mice were $KLRG\text{-}1^{Lo}/CD127^{Lo}$ (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 naïve 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 polycytokine-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 polycytokine-producing memory CD8 T cells, as compared to WT DCs. FLT3-induced DCs are believed to mimic the functional attributes of CD8+ DCs, which are known to play 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 [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 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 CDK inhibitory 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 (p27loxP) or a floxed Neomycin cassette inserted in the $p27^{Kip1}$ allele (p27STOP) have been described elsewhere [21]. To induce T cell-specific deletion of $p27^{Kip1}$, we crossed p27loxP 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 p27STOP mice with the CD4-Cre transgenic mice. Littermate WT mice were used as controls. Mice were infected intraperitoneally with 2×10^5 PFU of LCMV-Armstrong to induce an acute infection. Infectious LCMV was quantified by a plaque assay on Vero cells, as described previously [2]. For experiments involving derivation and transfer of bone marrow-derived dendritic cells, 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.

Quantitative RT-PCR

T cells and non-T cells were purified from spleens of WT, p27−/−, T-OFF and T-ON mice using the anti-CD90.2 MACS cell separation system (Miltenyi Biotec, Auburn CA). 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). Quantitative real-time PCR was performed using POWERSYBR Green Master Mix (Applied Biosystems, Foster City, CA) and data was 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 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). Samples containing 15μg of protein were resolved on a 12% SDS-PAGE. The $p27^{Kip1}$ protein in each sample was detected using a mouse primary antibody specific for $p27^{Kip1}$ (BD Bioscience, San Jose, CA), 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). Blots initially probed for $p27^{Kip1}$ were subsequently stripped and re-probed to detect β-Actin (Sigma-Aldrich, St. Louis MO) to serve as a loading control.

Flow cytometry

Single-cell suspensions of cells from spleen, liver or peripheral blood were prepared as previously described [2]. Mononuclear cells were stained with D^b MHC class I tetramers, specific for the LCMV epitopes NP396-404 (NP396) and GP33-41 (GP33) [2]. In some experiments, cells were co-stained with anti-CD44, anti-LFA-1, anti-CD62L, anti-CD122, anti-CD27, anti-CD127 and anti-KLRG-1 antibodies. All antibodies were purchased from BD Biosciences, eBioscience (San Diego CA) or Southern Biotech (Birmingham AL). Cells were fixed in 2% paraformaldehyde (PFA) and analyzed with FACSCalibur or LSR II flow cytometer (BD Biosciences, Franklin Lakes NJ). For intracellular cytokine staining,

splenocytes were stimulated *in vitro* with LCMV epitope peptides in the presence of brefeldin A for 5 h. After culture, cells were stained for surface CD8 and intracellular gamma interferon (IFNγ), tumor necrosis factor α (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 preformed as previously described [31].

Derivation and transfer of bone marrow-derived dendritic cells

Bone marrow derived dendritic cells (DCs) were generated as previously described [40]. Briefly, bone marrow cells from WT and p27−/− mice were cultured in 10% RPMI containing 100ng/ml mouse FLT3L (Peprotech, Rocky Hill, NJ) for 9 days. LPS (500ng/ml, Sigma-Aldrich, St. Louis, MO) was then added for 24 hours to induce maturation. Maturation was assessed via flow cytometry and cells were pulsed with 2 μM of GP33 peptide for 2 hours. Cells were washed extensively, and 5×10^5 $CD11c^{+ve}$ mature peptide-pulsed DCs were administered to WT C57BL/6 mice by intravenous (I/V) injection.

Statistical analysis

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

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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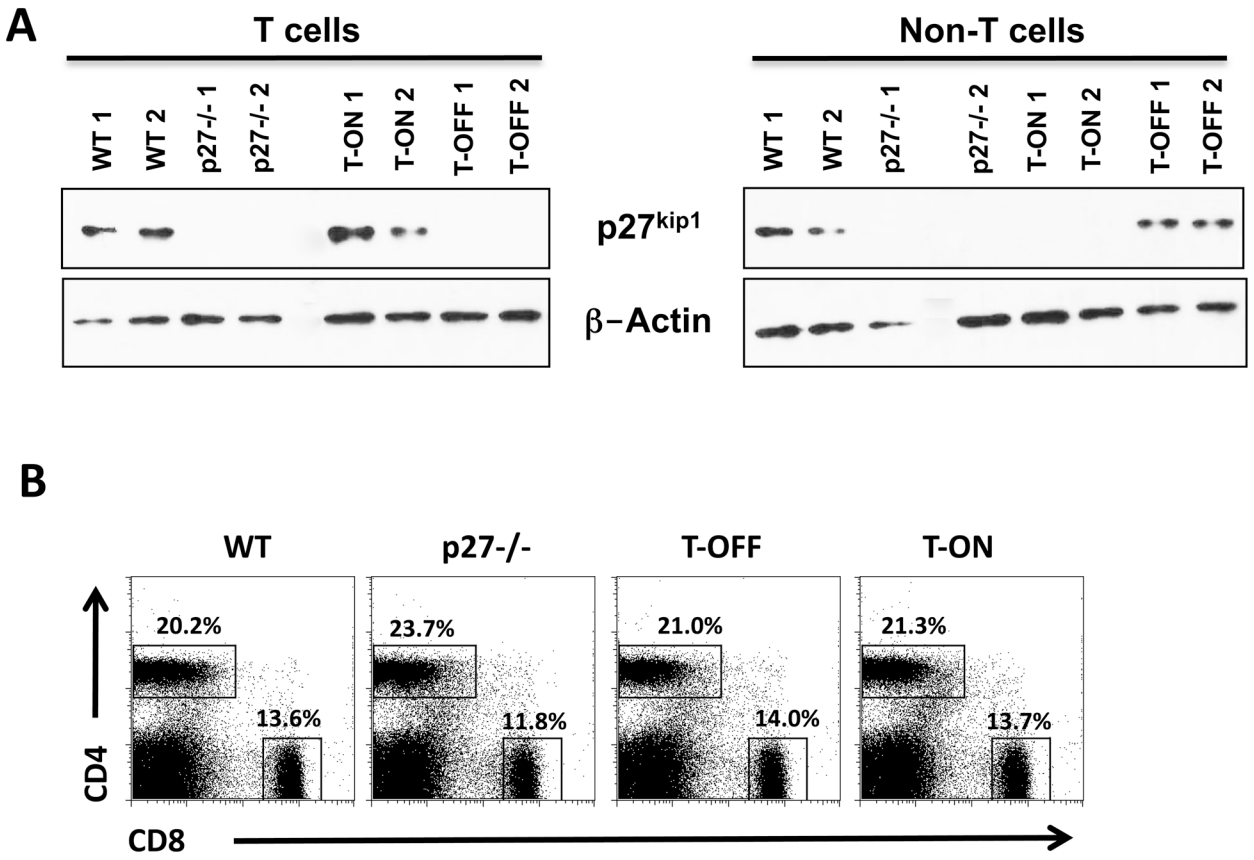

Figure 1. Characterization of T-OFF and T-ON mice

Splenocytes from naïve 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 β-Actin was employed as a loading control. Figure shows $p27^{Kip1}$ and β-Actin expression in T cells and non-T cells from two representative mice for each group. (b) Splenocytes from naïve 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.

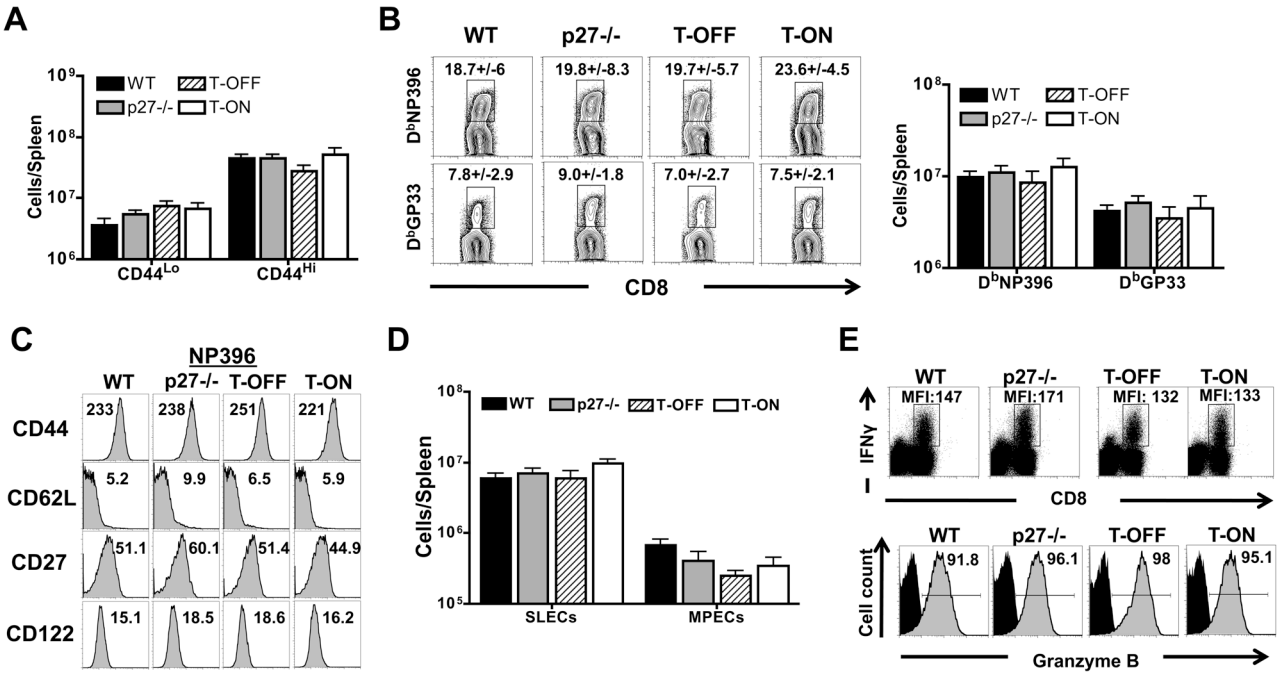

Figure 2. Ablation of p27^{Kip1} in T cells or non-T cells does not affect the clonal expansion, phenotype or function of effector CD8 T cells

(a) WT, p27−/−, T-OFF and T-ON mice were infected with LCMV and at 8 days PI, splenocytes were stained with anti-CD8 and anti-CD44. (b) To visualize LCMV-specific CD8 T cells at day 8 PI, splenocytes from WT, p27−/−, T-OFF and T-ON mice were stained anti-CD8 and D^b tetramers. Representative dot plots show percentages of D^b/NP396- (top) and D^b/GP33-(bottom) specific CD8 T cells, and are gated on total CD8 T cells. Bar graphs represent the total numbers of virus-specific CD8 T cells. (c) At day 8 PI, splenocytes were stained with anti-CD8 and D^b/NP396 tetramers in conjunction with anti-CD44, anti-CD62L, anti-CD27 and anti-CD122 antibodies. Representative histograms are gated on D^b/NP396 tetramer positive CD8 T cell populations from WT, p27−/−, T-OFF and T-ON mice. The numbers on the histograms represent the MFI for the indicated protein. (d) On day 8 PI, total splenocytes were stained with D^b/NP396 tetramer, anti-CD8, anti-CD127 and anti-KLRG-1, and the total number of SLECs (KLRG-1^{HI}/CD127^{LO}) and MPECs (CD127^{HI}/KLRG-1^{LO}) 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 TON mice were stimulated with NP396 peptide for 5 hours 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. (e) 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 3 independent experiments with 3–5 mice/group/experiment. Error bars represent the SEM and * indicates statistical significance at P<0.05.

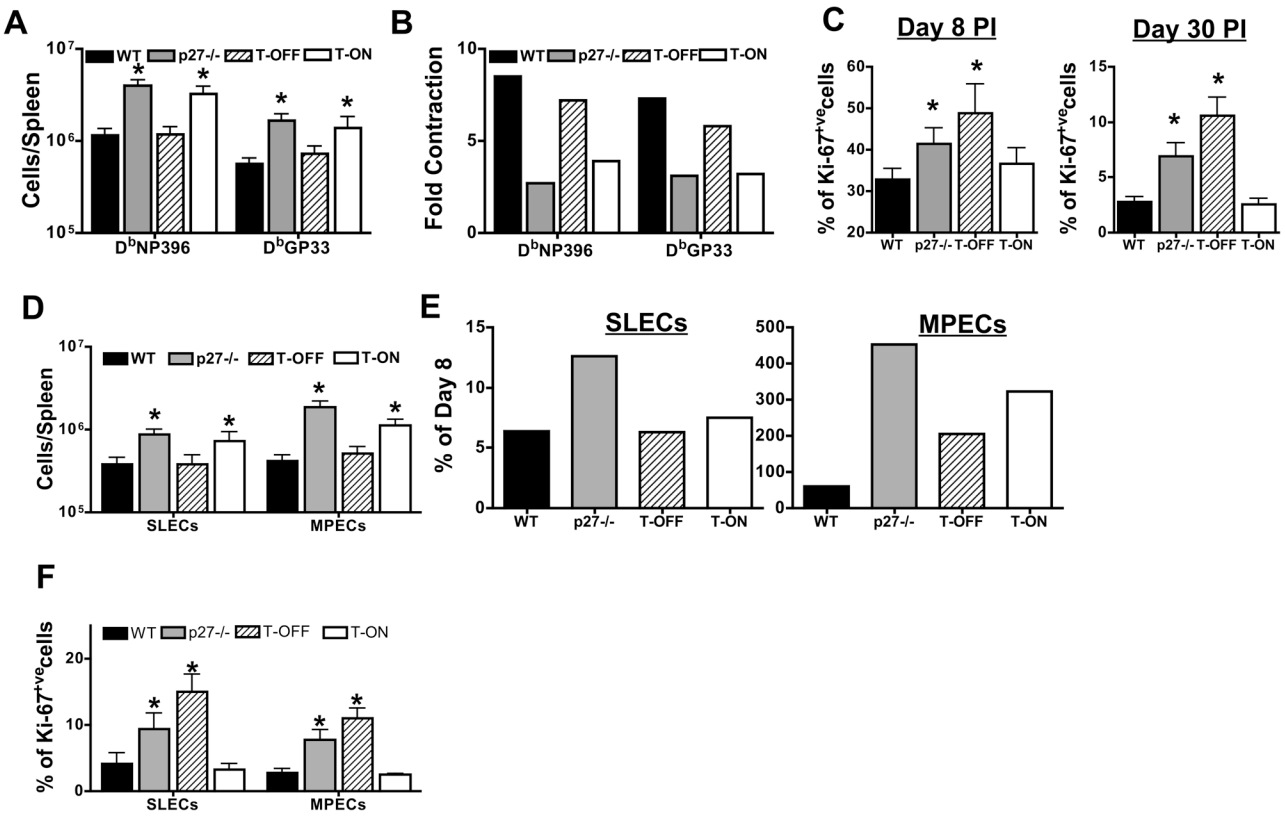

Figure 3. Contraction of effector CD8 T cells is regulated by p27^{Kip1} by T cell-extrinsic mechanisms independent of proliferation

(a) At day 30 PI, splenocytes fro 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 day 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 (KLRG-1^{HI}/CD127^{LO}) and MPECs (CD127^{HI}/KLRG-1^{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 contraction, we calculated the percentages of SLECs and MPECs from day 8 PI that survived until day 30 PI. (f) The percentage of Ki67^{+ve} cells amongst 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 amongst NP396-specific CD8 T cell effector subsets. Data are from 3 independent experiments with 3–5 mice/group/experiment. Error bars represent the SEM and * indicates statistical significance at $P<0.05$.

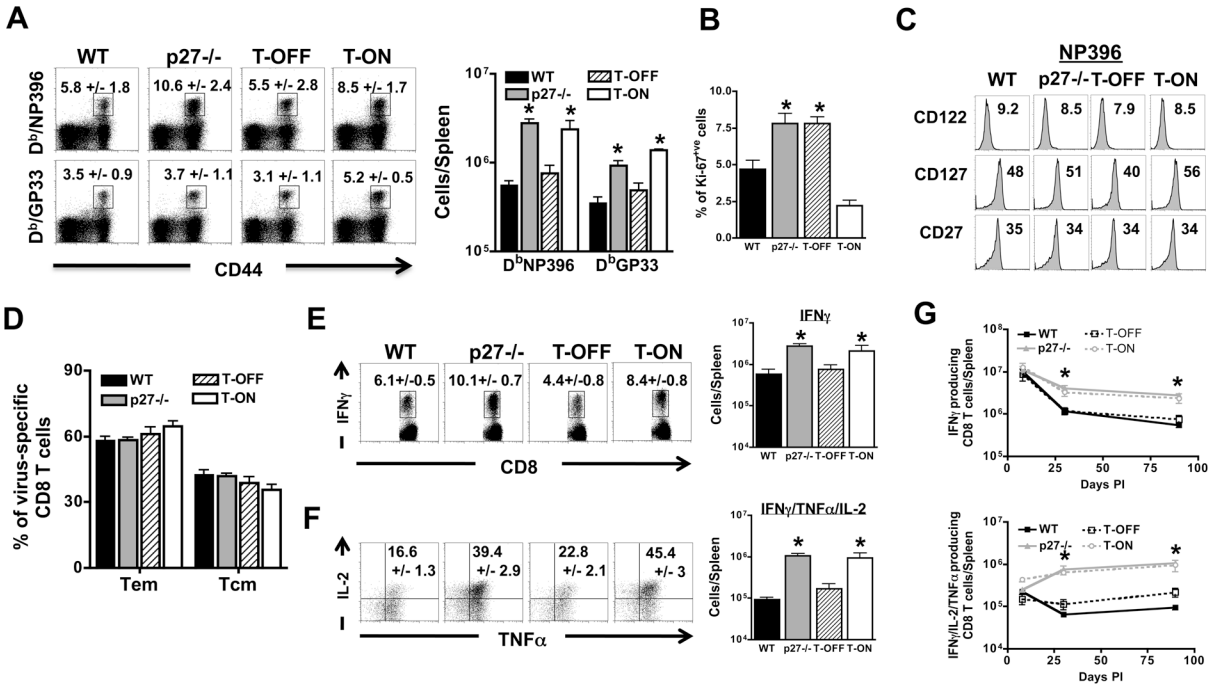

Figure 4. Deletion of p27^{Kip1} in non-T cells improves the quantity and quality of memory CD8 T cells

(a) At day 90 PI, splenocytes from WT, p27−/−, T-OFF and T-ON mice were stained with anti-CD8 and D^b tetramers. Representative FACS plots show staining for D^b/NP396- (top) and D^b/GP33- (bottom) specific CD8 T cells. Plots are gated on total CD8 T cells and the numbers represent the percentages of epitope-specific CD8 T cells of total CD8 T cells. Plots on the right show quantified flow cytometry data of tetramer-specific CD8 T cells in spleens of the WT, p27−/−, T-OFF and T-ON mice. (b) At day 90 PI, splenocytes from WT, p27−/−, T-OFF and T-ON mice were stained with anti-CD8, D^b/NP396 tetramers and anti-Ki67. Data is expressed as the percentages of Ki-67 positive cells amongst NP396-specific CD8 T cells. (c) Expression levels of cell surface proteins on LCMV-specific CD8 T cells. Splenocytes from WT, p27−/−, T-OFF and T-ON mice were stained with D^b/NP396, anti-CD8, anti-CD122, anti-CD27 or anti-CD127. Numbers represent the MFI for the NP396-specific CD8 T cell population from WT, p27−/−, T-OFF and T-ON mice. (d) Splenocytes from WT, p27−/−, TOFF and T-ON mice were isolated on day 90 PI, and stained with anti-CD8, anti-CD62L and D^b tetramers. Bar graph shows the percentages of effector memory (Tem [CD62L^{low}]) and central memory (Tcm [CD62L^{high}]) NP396-specific memory CD8 T cells from WT, p27−/−, T-OFF and T-ON mice. (e and f) At day 90 PI, splenocytes from WT, p27−/−, T-OFF and T-ON mice were stimulated with NP396 peptide for 5 hours. Following surface staining with anti-CD8, intracellular staining for IFNγ, IL-2 and TNFα was performed. (e) Representative FACS plots are gated on total CD8 T cells and show percentages of NP396-specific CD8 T cells that were positive for IFNγ. Bar graph shows the total number of NP396-specific IFNγ-producing CD8 T cells. (f) Representative FACS plots show NP396-specific CD8 T cells that produced IFNγ, IL-2 and TNFα (triple-cytokine producers). FACS plots are gated on IFNγ-producing CD8 T cells. Numbers indicate the percentages of triple cytokine producing virus-specific CD8 T cells +/− SD. The bar graph

on the right indicates the total number of triple cytokine-producing NP396-specific CD8 T cells. (g) WT, p27−/−, T-OFF and T-ON mice were infected with LCMV and on days 8, 30 and 90 PI splenocytes were isolated and NP396-specific CD8 T cells were analyzed for production of IFNγ, IL-2 and TNFα by intracellular cytokine staining Graph on the top shows the total number of NP396-specific IFNγ producing CD8 T cells CD8 T cells. Bar graph in the bottom shows the total numbers of triple-cytokine- producing NP396-specific CD8 T cells. Data are from 3 independent experiments with 3–5 mice/group/experiment. Error bars represent the SEM and * indicates statistical significance at $P<0.05$.

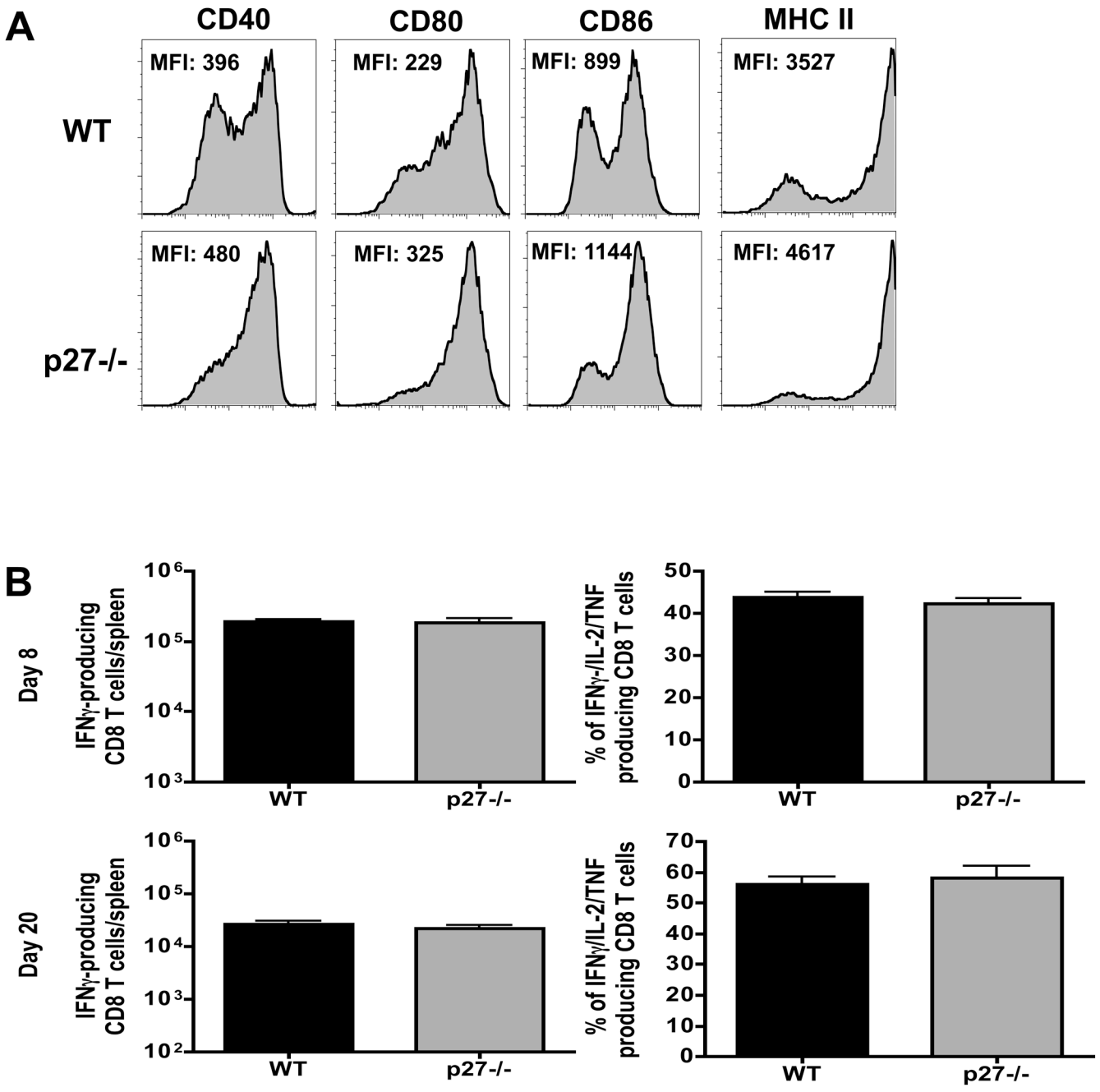

Figure 5. Priming of memory CD8 T cells by WT and p27−/− BMDCs

Bone marrow-derived DCs (BMDCs) were generated by culturing bone marrow cells from WT or p27 −/− mice with FL3TL. (a) Maturation of BMDCs was induced by stimulation with LPS for 24 hours. Maturation of DCs was assessed by staining cells with anti-CD11c, anti-CD80, anti-CD86 and anti-MHC II. Histograms are gated on CD11c positive cells. Numbers show the MFI for the indicated protein. (b) Bone marrow-derived DCs from WT or p27−/− mice were matured with LPS, pulsed with GP33 peptide and transferred into WT Ly5.2/C57BL/6 mice. At days 8 and 20 post-transfer, splenocytes were stimulated with GP33 peptide for 5 hours followed by staining with anti-CD8, anti-IFNγ anti-TNFα and

anti-IL-2. Graphs show the total numbers of GP33-specific CD8 T cells that produced IFNγ (left) or percentages of IFNγ-producing cells that also produced, TNFα and IL-2 (right). Error bars represent the SEM and * indicates statistical significance at $P<0.05$.