

# Memory CD8<sup>+</sup> T cells exhibit increased antigen threshold requirements for recall proliferation

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**A hallmark of immunological memory is the ability of previously primed T cells to undergo rapid recall responses upon antigen reencounter. Classic work has suggested that memory T cells proliferate in response to lower doses of antigen than naive T cells and with reduced requirements for co-stimulation. In contrast to this premise, we observed that naive but not memory T cells proliferate in vivo in response to limited antigen presentation. To reconcile these observations, we tested the antigen threshold requirement for cell cycle entry in naive and central memory CD8<sup>+</sup> T cells. Although both naive and memory T cells detect low dose antigen, only naive T cells activate cell cycle effectors. Direct comparison of TCR signaling on a single cell basis indicated that central memory T cells do not activate Zap70, induce cMyc expression, or degrade p27 in response to antigen levels that activate these functions in naive T cells. The reduced sensitivity of memory T cells may result from both decreased surface TCR expression and increased expression of protein tyrosine phosphatases as compared with naive T cells. Our data describe a novel aspect of memory T cell antigen threshold sensitivity that may critically regulate recall expansion.**

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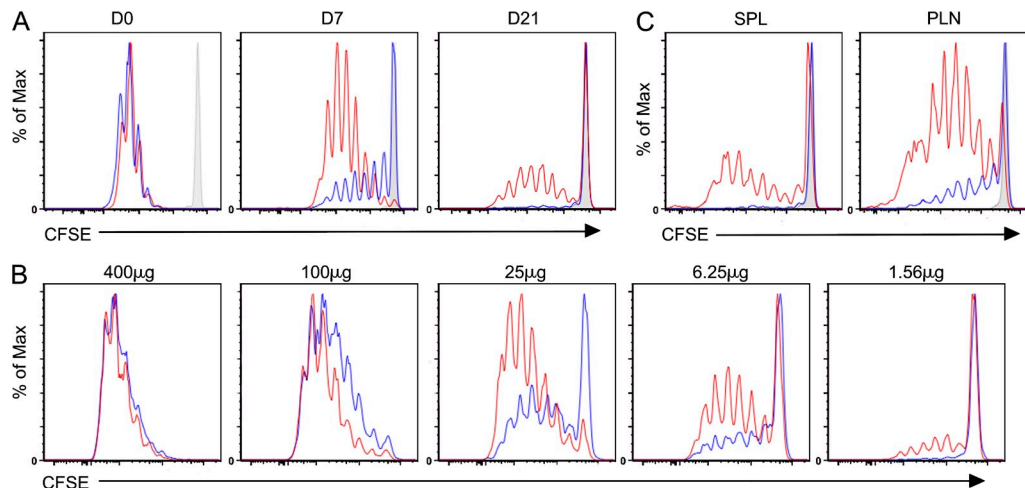
Abbreviations used: IC-OVA, immune complexed OVA; PTP, protein tyrosine phosphatase.

The ability of the adaptive immune system to respond more rapidly and effectively to pathogens that have been previously encountered is the basis of immunological memory. This attribute of CD8<sup>+</sup> T cell memory is primarily due to an estimated 5–100-fold increase in the frequency of antigen-specific cells after memory formation over that found in naive individuals (Ahmed and Gray, 1996). Additionally, evidence suggests that clonal competition during the expansion phase of T cell priming may increase the affinity of the resulting antigen-specific effector and memory CD8<sup>+</sup> T cell pool compared with the naive pool (Busch and Pamer, 1999; Zehn et al., 2009). Indeed, based on functionality, memory CD8<sup>+</sup> T cells appear to be more sensitive to TCR-mediated stimulation than naive cells. Multiple studies have observed that resting memory but not naive CD8<sup>+</sup> T cells can secrete cytokines and produce cytolytic effectors more rapidly than naive cells upon antigen encounter (Zimmermann et al., 1999; Veiga-Fernandes et al., 2000; Slifka and Whitton, 2001). Consistent with this ability, memory CD8<sup>+</sup> T cells show epigenetic changes at cytokine gene loci that are consistent with more rapid gene expression (Kersh et al., 2006; Northrop et al., 2006). In addition, memory

T cells redistribute their TCR into higher order oligomers that may increase antigen sensitivity (Kumar et al., 2011). Multiple phenotypic differences between naive and memory CD8<sup>+</sup> T cells have also been described that may influence TCR reactivity including up-regulation of adhesion molecules and increased surface expression of the IL-2R $\beta$  chain CD122 (Berard and Tough, 2002).

However, the characteristics ascribed to naive and memory T cells may have been influenced by the experimental systems used to test them. For example, although memory CD8<sup>+</sup> T cells reportedly proliferate in response to lower doses of antigen than naive T cells (Pihlgren et al., 1996; Curtsinger et al., 1998; London et al., 2000), little difference in peptide sensitivity was observed in the absence of exogenous IL-2 (Curtsinger et al., 1998; Zimmermann et al., 1999). Thus, the increased sensitivity of memory T cells to cytokine may be responsible for their superior response. Additionally, although some in vitro studies have found that memory CD8<sup>+</sup> T cells do not require CD28-mediated co-stimulation

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**Figure 1. Low antigen levels stimulate preferential proliferation of naive over memory CD8<sup>+</sup> T cells.** CFSE-labeled naive (red) and central memory (blue) OT-1 T cells were co-transferred into B6 mice at the indicated times after IC-OVA (A), or on the same day as immunization with OVA alone (B) or RIP-mOVA transgenic mice (C). CFSE dilution in the spleen or pancreatic lymph node (PLN) where indicated was determined 60 h later. Data are representative of a least 4 independent experiments with 2–3 mice/group.

to initiate recall expansion (Flynn and Müllbacher, 1996; Bachmann et al., 1999), B-7 expression appears to be necessary for recall expansion in vivo (Borowski et al., 2007; Boesteanu and Katsikis, 2009). These inconsistent data may be attributable to comparison of in vitro and in vivo results or inadequate analysis of the contribution of distinct memory CD8<sup>+</sup> T cell subsets. Extensive phenotyping of antigen-specific T cell responses has suggested that multiple markers may co-segregate with proliferative capacity. CD8<sup>+</sup> central memory T cells expressing CD44<sup>hi</sup>, CD62L<sup>hi</sup>, CD27<sup>hi</sup>, CXCR3<sup>hi</sup>, CD43<sup>lo</sup>, KLRG1<sup>lo</sup>, and CD127<sup>hi</sup> exhibit the most robust recall proliferation, whereas CD44<sup>hi</sup>, CD62L<sup>lo</sup>, CD27<sup>hi</sup>, CXCR3<sup>hi</sup>, CD43<sup>hi</sup>, KLRG1<sup>lo</sup>, and CD127<sup>hi</sup> effector memory T cells exhibit sustained cytotoxicity but poorer recall expansion (Wherry et al., 2003; Sallusto et al., 2004; Hikono et al., 2007; Olson et al., 2013).

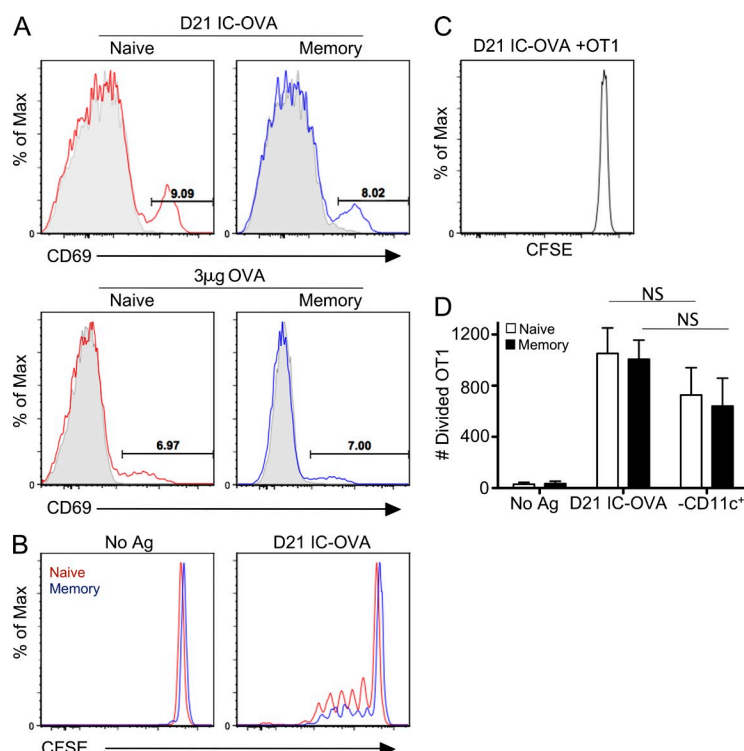
Intriguingly, it has been reported that after clearance of acute influenza infection, residual viral antigen presentation can drive proliferation and expansion of naive but not memory CD8<sup>+</sup> T cells of the same specificity (Belz et al., 2007; Khanna et al., 2008). This observation is in contrast to the expectation that memory T cells exhibit greater responsiveness than naive cells. It has been suggested that naive and memory T cells may respond to antigen presentation by distinct DC subsets or migrate to different areas of the lymph node (Belz et al., 2007; Kastenmüller et al., 2013). Currently, it remains unclear why residual, long-lived antigen does not stimulate memory T recall proliferation. To better understand the requirements for efficient proliferative recall expansion, we have compared the activation and proliferation of TCR transgenic CD62L<sup>hi</sup>CD44<sup>lo</sup> naive and CD62L<sup>hi</sup>CD44<sup>hi</sup> central memory CD8<sup>+</sup> T cells in multiple models of noninfectious antigen presentation at limiting levels in vitro and in vivo. Although we found that both naive and central memory cells received a

TCR stimulus and were activated by limiting levels of antigen presentation, only naive CD8<sup>+</sup> T cells entered cell cycle and expanded. This effect appeared T cell intrinsic, as naive T cells preferentially proliferated in response to limiting levels of peptide presented by multiple DC subsets. Direct comparison of naive and memory T cells indicated that resting memory CD8<sup>+</sup> T cells express more cyclin-dependent kinase inhibitor p27 and do not activate effectors of cell cycle progression or Zap70 upon low dose peptide stimulation, although no defect was observed at saturating concentrations. Additionally, we found that memory T cells expressed lower levels of surface TCR and higher levels of non-receptor tyrosine phosphatases involved in negative regulation of TCR signaling. Our data clearly indicates that, surprisingly, memory CD8<sup>+</sup> T cells actually exhibit a higher antigen threshold than naive CD8<sup>+</sup> T cells to stimulate cell cycle entry in vitro and in vivo.

## RESULTS

### Naive CD8<sup>+</sup> T cells preferentially proliferate in response to low dose antigen presentation

Prolonged antigen presentation after influenza infection can counterintuitively stimulate proliferation of naive but not memory CD8<sup>+</sup> T cells (Belz et al., 2007; Khanna et al., 2008). To determine if memory CD8<sup>+</sup> T cells respond to other long-lived antigens, we immunized B6 mice with immune-complexed OVA (IC-OVA) and at various times thereafter adoptively co-transferred purified CFSE-labeled CD44<sup>lo</sup>CD62L<sup>hi</sup>CD45.1<sup>+</sup> CD45.2<sup>+</sup> naive OT-1 and CD44<sup>hi</sup>CD62L<sup>hi</sup>CD45.1<sup>+</sup> central memory OT-1 T cells. 3 d after the T cell transfer, spleens and lymph nodes were isolated, and cell division was assessed by CFSE dilution. As expected, both naive and memory OT-1 cells proliferated extensively when transferred into mice that had been immunized with IC-OVA on the same day, with all transferred OT-1 cells having gone through more than six



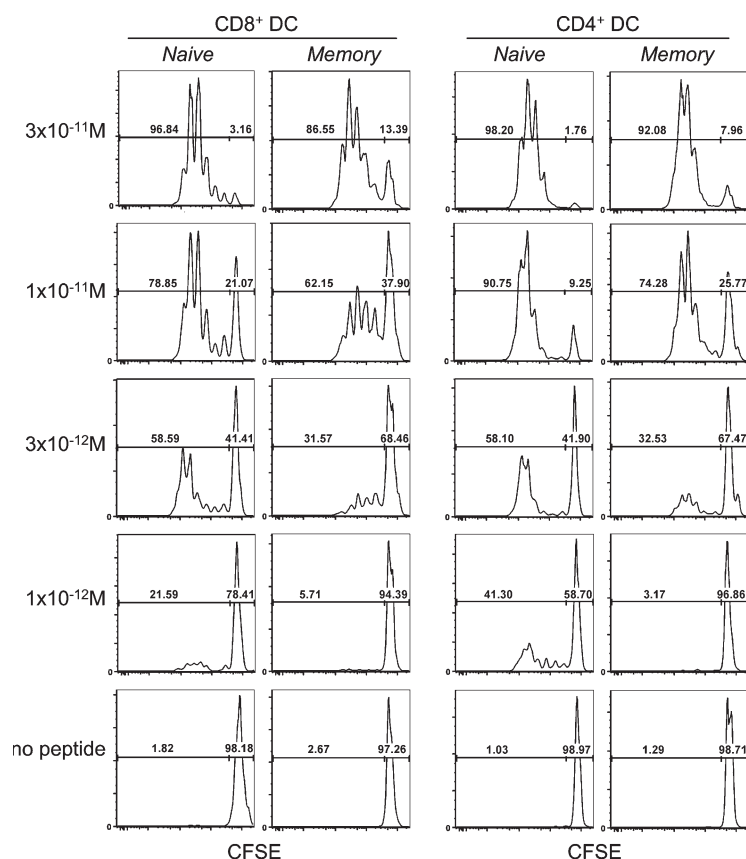
**Figure 2. Memory CD8<sup>+</sup> T cells receive TCR stimulation from prolonged antigen presentation and low dose immunization.** (A) Naive (red) and memory (blue) OT-1 T cells were stained for CD69 18 h after co-transfer into B6 mice immunized 21 d earlier with IC-OVA (top) or with 3 µg OVA (bottom). Data are representative of at least 3 independent experiments with 2–3 mice per group. (B–D) At 18 h after co-transfer of CFSE-labeled naive (red) and memory (blue) OT-1 T cells into mice immunized 21 d previously with IC-OVA, total splenocytes were cultured in the presence of rhIL-2 for 48 h. CFSE dilution was monitored by flow cytometry on congenic populations. Data are representative of at least 2 experiments with 2–3 mice/group. (C) Control freshly isolated CFSE-labeled OT-1 T cells were added to spleen suspensions isolated from day 21 IC-OVA immunized mice and cultured in the presence of rhIL-2 for 48 h. (D) Total numbers of naive and memory OT-1 T cells after ex vivo culture in rhIL-2 for 48 h with or without depletion of CD11c<sup>+</sup> cells ( $n = 6$ /group). Data were analyzed by Student's  $t$  test (NS,  $P > 0.05$ ). Error bars represent SEM for experiments performed in duplicate.

cell divisions (Fig. 1 A). When the OT-1 injection was delayed for 7 d after IC-OVA immunization, both naive and memory cells entered cell cycle, with the naive cells dividing more robustly. When the OT-1 T cell injection was delayed for 21 d after IC-OVA immunization, dramatic differences in naive and memory T cell division were observed. At this time point, whereas over half of the naive T cells had divided within 3 d, almost none of the memory population had entered cell cycle (Fig. 1 A). The failure of memory OT-1 to divide in response to long-lived IC-OVA antigen detected by naive cells in the same host was not due to competition for antigen-presenting cells, as separate transfer of naive and memory OT-1 into immunized mice gave the same result (unpublished data). These data confirm that IC-OVA-mediated prolonged antigen presentation does not stimulate memory CD8<sup>+</sup> T cell recall expansion.

To determine if prolonged antigen presentation after IC-OVA immunization provides unique signals to selectively stimulate proliferation of naive but not memory T cells, we tested if acute immunization with titrated amounts of soluble OVA protein alone would preferentially stimulate naive T cell expansion. To model the antigen levels encountered during prolonged presentation, we immunized mice with endotoxin-free OVA protein over an ~300-fold dose range at the same time as adoptive co-transfer of CFSE-labeled naive and memory OT-1 cells. 3 d after immunization and T cell transfer, spleens and LNs were harvested and OT-1 division was determined on the congenically marked naive and memory populations. We found that immunization with 400 µg of whole

OVA protein alone drove extensive division of both naive and memory OT-1 T cells (Fig. 1 B). Notably, however, as the dose of injected OVA decreased, we observed the preferential proliferation of naive T cells over memory T cells. In response to immunization with 1.56 µg OVA protein, approximately half of the naive OT-1 isolated 3 d after immunization had entered cell cycle, whereas memory OT-1 T cells showed nearly undetectable CFSE dilution. These data suggest that memory CD8<sup>+</sup> T cells divide poorly in vivo in response to low doses of antigen.

Although the endotoxin-free OVA protein was dialyzed to remove free peptide, immunization may transfer other contaminants that may influence T cell responsiveness. To determine if low level endogenous antigen presentation also stimulates preferential division of naive CD8<sup>+</sup> T cells, CFSE-labeled naive and memory OT-1 T cells were co-transferred into naive RIP-mOVA transgenic mice, which express a membrane-bound form of OVA in the pancreas. In the absence of any exogenous stimulation, a quarter of naive OT-1 T cells found in the spleen of RIP-mOVA transgenic mice underwent division within 3 d (Fig. 1 C). In contrast, none of the memory OT-1 T cells in the spleen had divided in the same animals. Increased division of both naive and memory T cells was observed in pancreatic lymph nodes. This is consistent with a higher level of antigen presentation in the nodes draining the site of transgene expression. These data from three different models further support the interpretation that, compared with memory T cells, naive CD8<sup>+</sup> T cells preferentially divide in vivo in response to limiting doses of antigen.



**Figure 3. All DC subsets stimulate preferential proliferation of naive CD8<sup>+</sup> T cells to limiting antigen dose in vitro.** Sorted splenic CD8<sup>α</sup><sup>+</sup> and CD4<sup>+</sup> DCs were peptide pulsed at the indicated dose for 30 min, washed, and cultured with either CFSE-labeled naive or memory OT-1 T cells. After 60 h, cell division was determined by flow cytometry. Representative profiles are shown. Data are representative of 2 experiments performed in duplicate.

### Memory cells receive a TCR stimulus from limiting levels of antigen presentation

That memory CD8<sup>+</sup> T cells do not proliferate after exposure to limited antigen presentation could be due to defective localization or interaction with antigen-presenting cells in vivo. To determine if nondividing memory CD8<sup>+</sup> T cells perceive or are ignorant of low-density antigens in vivo, we checked expression of T cell activation markers after co-transfer of naive and memory OT-1 cells into B6 mice immunized either with IC-OVA 21 d earlier or with 3 μg OVA on the same day. We observed CD69 up-regulation on a minor subset of both naive and memory cells 18 h after transfer, but not after transfer into unimmunized mice (Fig. 2 A). Additionally, we found no expression of the putative exhaustion marker PD-1 on either transferred T cell population, nor did PD-L1 blockade rescue memory OT-1 T cell division (unpublished data). These data suggest that both naive and memory CD8<sup>+</sup> T cells receive TCR stimulation from prolonged antigen presentation in vivo.

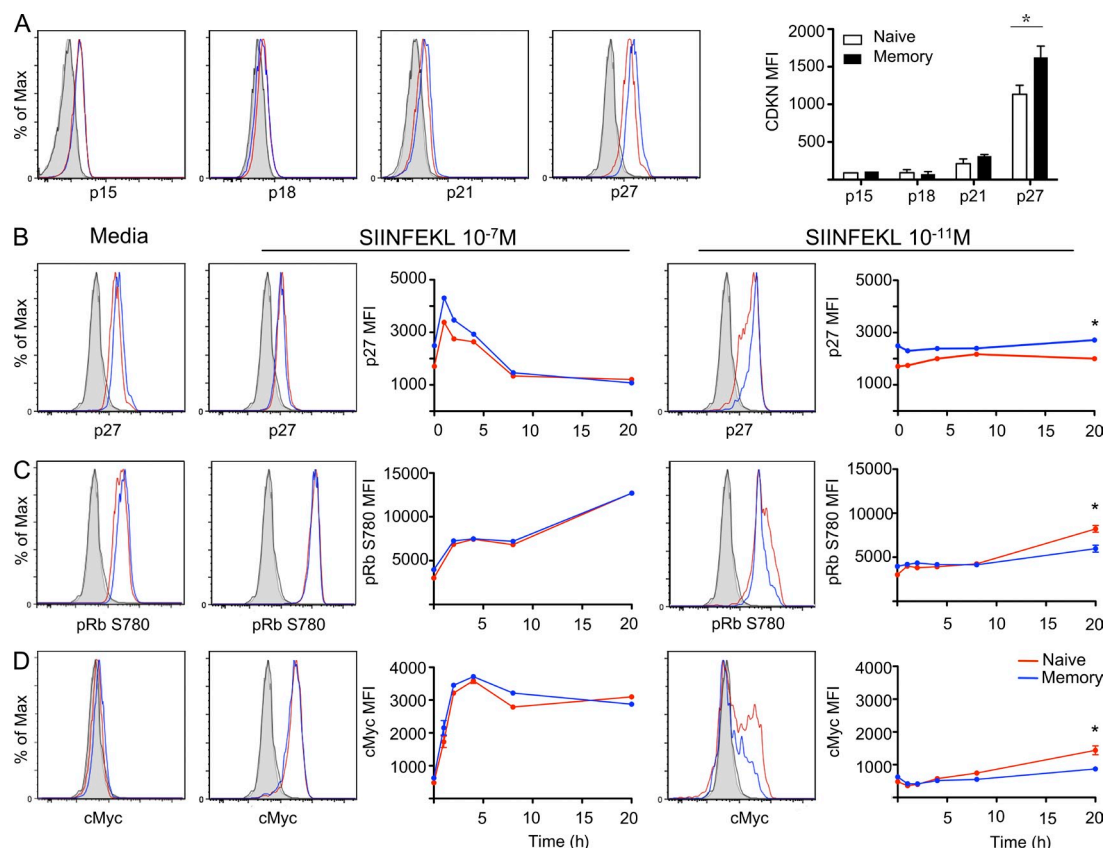
To confirm this conclusion, lymph node cell suspensions taken at 18 h after adoptive transfer of CFSE-labeled naive and memory OT-1 into day 21 IC-OVA recipients were cultured in vitro in the presence of exogenous IL-2 for an additional 48 h before assessing cell division (Fig. 2 B). We found no division when CFSE-labeled naive and memory OT-1 T cells had been transferred into unimmunized B6 recipients. However, after short-term transfer into mice immunized with

IC-OVA 3 wk earlier, about half of both naive and memory OT-1 cells divided ex vivo in the presence of exogenous IL-2. As controls, freshly isolated OT-1 cells added to the spleen or lymph node suspensions did not divide, suggesting that antigen presentation during the 48-h in vitro culture was not responsible for driving division (Fig. 2 C). Similarly, no change in proliferation was observed when CD11c<sup>+</sup> cells were depleted from the cell suspensions before culture (Fig. 2 D). Thus, these data indicate that both naive and memory CD8<sup>+</sup> T cells receive a TCR stimulus in vivo from low dose antigen presentation, but memory CD8<sup>+</sup> T cells do not enter cell cycle.

### Naive CD8<sup>+</sup> T cells preferentially proliferate regardless of DC subset

In multiple models of OVA immunization, CD8<sup>α</sup><sup>+</sup> DCs have been found to mediate antigen cross-presentation (den Haan and Bevan, 2002; Benke et al., 2006). Previous work has suggested that memory CD8<sup>+</sup> T cells may require interaction with this specific DC subset to stimulate proliferative recall expansion (Belz et al., 2007). To determine if specific conventional DC subsets influence the preferential proliferation of naive CD8<sup>+</sup> T cells in response to low dose antigen, we purified splenic CD8<sup>α</sup><sup>+</sup> and CD4<sup>+</sup> DCs by sorting. Isolated DCs were peptide pulsed and co-cultured for 60 h with CFSE-labeled naive or central memory OT-1 T cells without the addition of exogenous IL-2. We found that this assay was exquisitely sensitive to low antigen density with a linear response





**Figure 4. Central memory CD8<sup>+</sup> T cells do not activate effectors of cell cycle progression in response to low antigen dose stimulation.**

(A) Naive (red) and central memory (blue) resting OT-1 T cells were stained for nuclear expression of cyclin-dependent kinase inhibitors. Data are representative of at least 3 independent experiments performed in duplicate. Graph (right) shows the average MFI values ( $n = 6$ ). Data were analyzed by Student's *t* test (\*,  $P > 0.05$ ). Error bars represent SEM for all experiments performed. (B–D) Splenocytes containing congenically marked naive (red) and central memory (blue) OT-1 T cells were stimulated with high and low dose peptide, and OT-1 T cells were stained for p27 CDKN1B (B), phosphorylated Rb S780 (C), or cMyc (D). Histograms are representative of staining at 20 h in at least 3 independent experiments performed in duplicate. Nuclear expression over time as determined by mean fluorescence intensity is graphed for each antigen dose ( $n = 6$ ). Data were analyzed by Student's *t* test (\*,  $P < 0.05$ ).

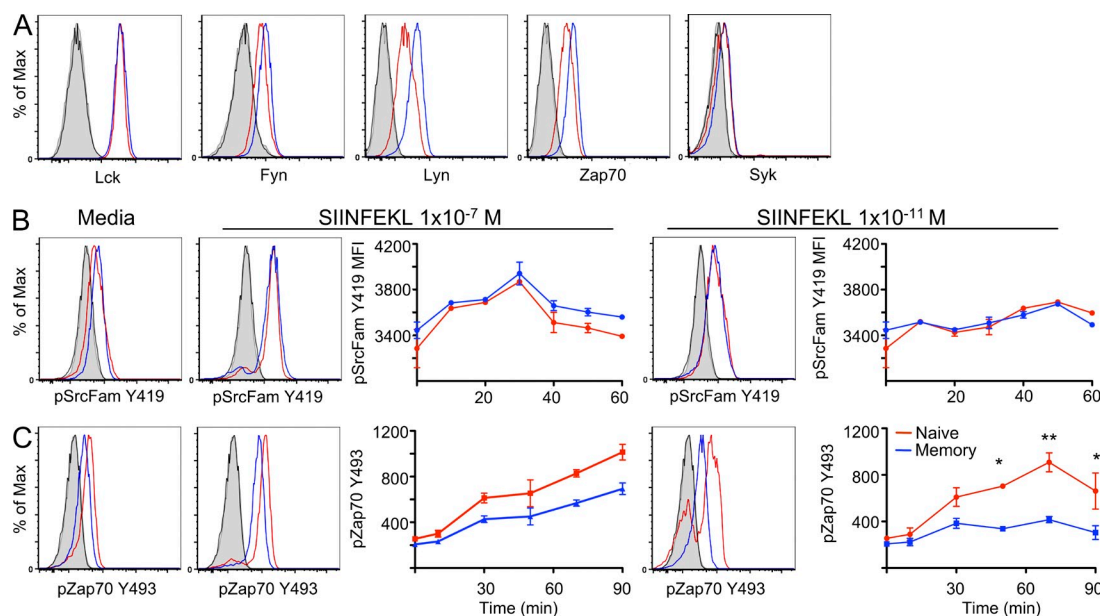
range between 1 pM and 0.1 nM SIINFEKL peptide. However, all peptide doses within this range stimulated greater proliferation of naive OT-1 cells than central memory OT-1 cells regardless of the DC subset-presenting antigen (Fig. 3). Intriguingly, similar results have also been observed using bone marrow–derived DCs (Wakim and Bevan, 2011). In contrast to previous results characterizing the sensitivity of naive and memory CD8<sup>+</sup> T cells in the presence of IL-2 (Pihlgren et al., 1996; Curtsinger et al., 1998; London et al., 2000), these data imply that T cell–intrinsic mechanisms likely control the threshold antigen density required for cell cycle entry.

#### Memory CD8<sup>+</sup> T cells do not activate effectors of cell cycle progression in response to low dose Ag stimulation

Memory CD8<sup>+</sup> T cells may exhibit a higher threshold for cell cycle entry due to increased expression of cyclin-dependent kinase inhibitors compared with naive cells. Indeed, microarray analysis has previously indicated this to be the case (Latner et al., 2004). In contrast, comparison of naive and memory CD8<sup>+</sup> T cells by Western blot analysis has indicated that only

p18 CDKN2A is differentially regulated (Veiga-Fernandes and Rocha, 2004). To compare protein expression on a per cell basis, we stained resting naive and central memory OT-1 T cells for various cyclin-dependent kinase inhibitors. We observed limited expression of p15 CDKN2B, p18 CDKN2A, and p21 CDKN1A. However, we found that expression of p27 CDKN1B was slightly augmented in resting central memory OT-1 T cells (Fig. 4 A). In contrast to previous microarray and Western blot experiments, these data indicate resting memory T cells express higher levels of p27 compared with naive T cells, which may restrict cell cycle entry.

Regulated by ubiquitin-mediated proteolysis, p27 CDKN1B can bind CDK4/CDK6–cyclin D and CDK2–cyclin E complexes to prevent G0–G1/S cell cycle progression (Sherr and Roberts, 1999). To determine how p27 expression is controlled in response to antigen stimulation, we examined p27 expression after activation in mixed cultures of naive and central memory T cells (Fig. 4 B). Surprisingly, strong activating signals from high dose peptide stimulation increased p27 expression at early time points. At later time points, elimination



**Figure 5. Central memory CD8<sup>+</sup> T cells do not activate Zap70 in response to low antigen dose stimulation.** (A) Naive (red) and central memory (blue) resting OT-1 T cells were stained for intracellular expression of Src family and Syk family kinases. Data are representative of more than 3 independent experiments. (B and C) Splenocytes containing congenic naive (red) and memory (blue) OT-1 cells were stimulated by addition of either high or low dose SIINFEKL peptide, and then stained for total phosphorylated Src family kinase (B), phosphorylated Zap70/Syk (C), or isotype control (gray). Histograms are representative of staining at the peak of the response in at least 3 independent experiments performed in duplicate. Expression over time as determined by mean fluorescence intensity is graphed for each antigen dose ( $n = 6$ ). Data were analyzed by Student's *t* test (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ). Error bars represent SEM for experiments performed in duplicate.

of p27 was observed in both naive and memory OT-1 T cells consistent with protein degradation. In contrast, in response to low dose peptide stimulation only naive OT-1 T cells down-regulated p27 expression at 20 h after stimulation, whereas expression of p27 did not change in memory OT-1 cells. These data indicate that the strength of TCR signaling in naive and memory T cells controls p27 degradation. Considering the increased p27 expression observed in memory CD8<sup>+</sup> T cells, this implies that memory cells may require a greater stimulus to bring p27 expression levels down to those observed in naive T cells.

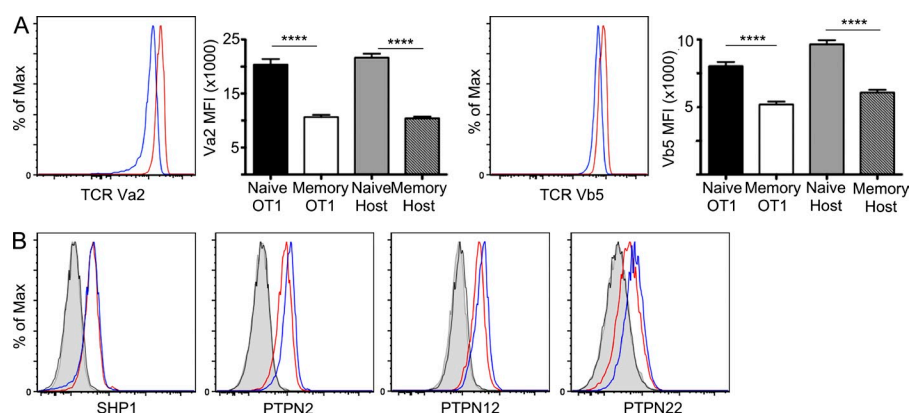
To confirm that effectors of cell cycle progression are not activated in memory cells in response to low dose antigen, we tested for phosphorylation of Rb. Previous work has indicated phosphorylation of Rb at S780 requires cyclin D (Geng et al., 2001), thus indicating CDK4/CDK6 activation. In response to high dose SIINFEKL peptide stimulation, we observed a steady increase in Rb pS780 in both naive and central memory OT-1 T cells (Fig. 4 C). However, in response to low dose peptide stimulation Rb pS780 was only observed to increase in naive and not memory T cells. Interestingly, this form of phosphorylated Rb appeared in response to low dose stimulation in naive T cells at a similar time point as p27 CDKN1B down-regulation. These data indicate that effectors of cell cycle progression are not stimulated in memory T cells in response to low density antigen presentation.

cMyc is a master transcriptional regulator of cell cycle progression and directly controls expression of cyclins (Dang,

1999) and Cul1, a critical component of the SCF-SKP2 complex involved in p27 degradation (O'Hagan et al., 2000). To further confirm that effectors of cell cycle progression are not activated in memory T cells in response to low dose antigen, we tested for cMyc expression. We found that maximal cMyc expression was stimulated by high dose peptide in both naive and memory OT-1 T cells within 4 h of stimulation (Fig. 4 D). In contrast, cMyc expression in response to low dose antigen stimulation was observed only at later times and primarily in naive T cells. Interestingly, much greater cMyc expression in both naive and memory T cells was induced with high dose peptide stimulation than low dose. These data clearly indicate that strength of TCR signaling in naive and memory T cells controls cMyc expression and that naive T cells are more sensitive to TCR signals that result in cMyc activation.

### Memory CD8<sup>+</sup> T cells do not activate Zap70 in response to low dose Ag stimulation

As our results suggested that central memory T cells poorly activate distal effectors of TCR signaling in response to weak antigen stimulus, we investigated activation of more proximal TCR signaling components. Biochemical comparisons have identified expression of unique phosphoproteins and augmented lipid raft domains in resting memory T cells compared with naive T cells (Farber et al., 1997; Kersh et al., 2003), suggesting that memory cells may transduce TCR signals



**Figure 6. Central memory CD8<sup>+</sup> T cells express less TCR and more PTP than naive.** (A) Surface expression of TCR Vα2 and TCR Vβ5 on resting CD26L<sup>hi</sup>CD44<sup>lo</sup> naive (red) and CD26L<sup>hi</sup>CD44<sup>hi</sup> central memory (blue) OT-1 and polyclonal B6 naive and central memory T cells was determined. Data are representative of at least 3 independent experiments with 3–5 mice/group. Graphs show the average MFI values ( $n > 5$ /group). Data were analyzed by Student's *t* test (\*\*\*\*,  $P < 0.0001$ ). Error bars represent SEM for all experiments performed. (B) Intracellular expression of SHP-1, PTPN2, PTPN12, and PTPN22 on naive (red) and memory (blue) OT-1 T cells was determined. Data are representative of at least 3 independent experiments performed in duplicate.

differently than naive T cells. To confirm expression of critical TCR signaling intermediates in naive and memory CD8<sup>+</sup> T cells, we tested for the expression of Src and Syk family members in naive and central memory OT-1 T cells. Using monoclonal antibodies specific for individual Src family kinases, we confirmed previous observations (Kersh et al., 2003) that memory T cells express similar levels of lck expression but increased expression of fyn kinase on a per cell basis compared with naive cells (Fig. 5 A). Intriguingly, we also observed increased expression of lyn kinase in central memory T cells, a Src family member involved in negative regulation of BCR signaling (Xu et al., 2005). In addition, although both naive and memory CD8<sup>+</sup> cells expressed negligible levels of Syk kinase, much higher levels of Zap70 were observed in central memory OT-1 cells as compared with naive cells. These data clearly indicate that resting naive and central memory CD8<sup>+</sup> T cells differentially express proximal TCR signaling components.

To determine if naive and central memory CD8<sup>+</sup> T cells activate proximal TCR signaling components similarly, we stained for phosphorylated total Src family and Zap70 in mixed cultures of congenically marked naive and central memory OT-1 T cells stimulated with peptide for various times. Although central memory T cells expressed alternate Src family kinase members, total Src family activation after peptide stimulation was similar in both naive and memory T cells after high and low dose peptide stimulation, as determined by phosphorylation of all Src family members at residues corresponding to Y419 at the active site (Fig. 5 B). In contrast, central memory OT-1 T cells expressed higher levels of total Zap70 than naive cells but showed reduced Zap70 activation as measured by phosphorylation at Y493 within the activation loop (Fig. 5 C). At low peptide doses, Zap70 activation in memory CD8<sup>+</sup> T cells was barely detectable above the level in resting cells (Fig. 5 C). These data indicate that memory CD8<sup>+</sup> T cells activate Zap70 less efficiently than naive cells in response to low dose peptide stimulation.

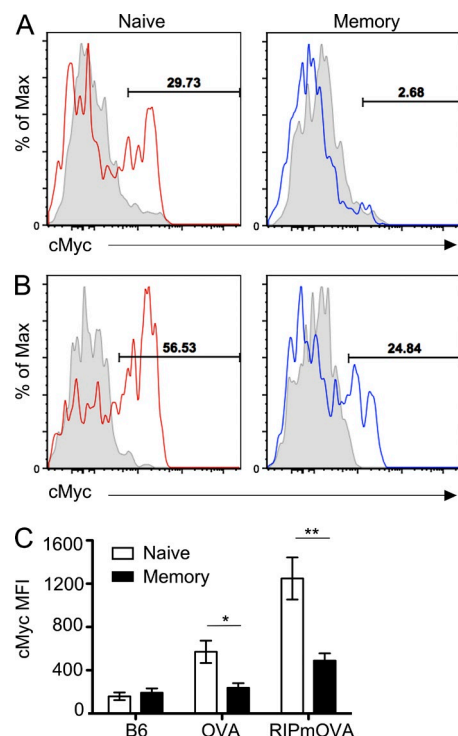
### Memory CD8<sup>+</sup> T cells express lower levels of TCR and more protein tyrosine phosphatases (PTPs)

Robust Zap70 activation is dependent on its TCR association mediated by CD3ζ ITAM phosphorylation (Wang et al., 2010). To determine if the distribution of the OT-1 TCR was similar in resting naive and central memory OT-1 T cells, we stained for cell surface expression of the transgenic Vα2 and Vβ5 TCR chains. Similar to previous observations (Curtsinger et al., 1998; Kersh et al., 2003), we found that total TCR expression was approximately twofold reduced in central memory OT-1 cells as compared with naive cells (Fig. 6 A). Similar results for TCR Vα2 and Vβ5 expression were observed even in the polyclonal, endogenous CD62L<sup>hi</sup>CD44<sup>lo</sup> naive, and CD62L<sup>hi</sup>CD44<sup>hi</sup> central memory CD8<sup>+</sup> T cell pool of the adoptive host. These data suggest that central memory CD8<sup>+</sup> T cells express reduced levels of TCR on their surface compared with their naive counterparts.

The threshold for TCR activation is also modulated by PTPs (Rhee and Veillette, 2012). To determine the distribution of nonreceptor PTPs known to regulate TCR activation, we stained resting naive and memory OT-1 cells for intracellular PTP expression. Although we found no difference in SHP-1 expression, resting central memory T cells exhibited increased expression of PTPN2 (TC-PTP), PTPN12 (PTP-PEST), and PTPN22 (Lyp or PEP; Fig. 6 B). These data indicate that central memory T cells express higher levels than naive T cells of multiple PTPs known to negatively regulate TCR signal transduction (Hasegawa et al., 2004; Davidson et al., 2010; Wiede et al., 2011).

### Memory CD8<sup>+</sup> T cells activated in vivo by low dose antigen do not up-regulate cMyc

As central memory T cells poorly activate TCR signaling in vitro in response to weak antigen stimulation, we tested if similar mechanisms control the proliferative response of memory T cells to low-density antigen in vivo. We co-transferred naive and central memory OT-1 T cells into mice that either received low dose endotoxin-free OVA immunization or



**Figure 7. Memory CD8<sup>+</sup> T cells activated in vivo to low dose antigen do not up-regulate cMyc.** Naive (red) and memory (blue) OT-1 cells were co-transferred into B6 mice immunized with 3 µg OVA (A), Rip-mOVA transgenic mice (B), or untreated control B6 (gray). At 20 h after transfer, OT-1 cells were stained for CD69 and cMyc on congenic populations. Expression of cMyc on CD69<sup>+</sup> activated naive and central memory OT-1 was quantified by flow cytometry. Data are representative of 3 experiments with 2–3 mice/group. (C) Mean fluorescence intensity of cMyc staining on CD69<sup>+</sup> congenic naive and memory OT-1 is graphed ( $n = 5$ /group). Data were analyzed by Student's *t* test (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ). Error bars represent SEM for all experiments performed.

expressed the RIPmOVA transgene. At ~20 h after T cell transfer, lymph node cells were isolated and cMyc expression was determined on CD69<sup>+</sup> activated naive and memory OT-1 T cells. Although both populations were similarly activated upon antigen exposure in vivo as determined by CD69 expression, only naive and not memory OT-1 cells expressed cMyc in response to protein immunization (Fig. 7, A and C) or to the presence of OVA in the RIPmOVA recipients (Fig. 7, B and C). These results correlate with the absence of memory T cell proliferation that we observed previously in these model systems (Fig. 1, B and C). Thus, in two noninflammatory models of antigen presentation, activated memory CD8<sup>+</sup> T cells receive blunted TCR signals in vivo resulting in less cMyc expression and reduced proliferation.

## DISCUSSION

Despite recent progress characterizing the development, phenotype and function of CD8<sup>+</sup> memory T cells, the requirements to stimulate proliferative recall responses remain poorly understood. We sought to understand the activation of naive

and central memory CD8<sup>+</sup> T cells in response to limiting levels of antigen presentation. Herein, we report that although both naive and central memory CD8<sup>+</sup> T cells were stimulated in vivo by low dose and prolonged antigen presentation, only naive and not memory OT-1 T cells entered cell cycle and proliferated. These data indicate that although memory T cells correctly localize in the lymphoid organs to receive TCR stimulation, they do not divide in response to low-density antigen presentation. The preferential proliferation of naive T cells was not dependent on the type of DC-presenting antigen, as naive CD8<sup>+</sup> T cells preferentially proliferated in response to low dose peptide stimulation in the absence of exogenous IL-2 regardless of the type of APC. Similar observations of preferential expansion of naive T cells as compared with memory cells have recently been reported even in response to acute infection (Jellison et al., 2012; Martin et al., 2012). However, during an infectious challenge it is not possible to uncouple the effects of inflammatory mediators from antigen load. An advantage of our noninfectious system is the ability to separately manipulate antigen dose from inflammatory signals that also influence memory T cell reactivity (Raué et al., 2013; Richer et al., 2013). Our findings clearly show that naive T cells proliferate in response to lower levels of antigen than memory T cells in vitro and in vivo. These results directly challenge previous conclusions that the antigen threshold for activation of memory T cells is lower than that of naive T cells (Pihlgren et al., 1996; Curtsinger et al., 1998; London et al., 2000).

Although most studies argue that memory CD8<sup>+</sup> T cells exhibit increased antigen sensitivity compared with naive cells, surprisingly little information actually exists on the activation of signaling intermediates in primary CD8<sup>+</sup> T cells stimulated with physiological levels of antigen (Zehn et al., 2012). Through direct comparison of TCR transgenic cells of the same number and specificity, we avoided the known complications of precursor frequency and TCR avidity on CD8<sup>+</sup> T cell activation and proliferation (Badovinac et al., 2007; Zehn et al., 2009). Critically, we observed reduced Zap70 activation and cMyc induction in memory CD8<sup>+</sup> T cells as compared with naive T cells specifically in response to low-density ligand stimulation. Our findings expand upon previous studies that found no difference in Zap70 activation in primary naive and memory CD8<sup>+</sup> T cells in response to strong anti-CD3-mediated stimulation (Kersh et al., 2003). Our results highlight the importance that the increased sensitivity a flow cytometry-based approach allows to directly compare primary naive and memory T cells activated in the same culture on a per cell basis. Careful phenotyping of input populations before analysis facilitated informative comparison of naive T cell responses specifically to central memory T cells, previously characterized to exhibit the greatest proliferative potential (Sallusto et al., 2004). Intriguingly, central memory T cells exhibited slightly weaker Zap70 activation than naive cells even in response to strong stimuli within the time course of our study. This likely correlates with the reduced TCR expression that we observed on memory T cells, as Zap70



activation is dependent on TCR association (Wang et al., 2010). Our data confirms previous studies indicating that Zap70 activation is required for TCR-mediated peripheral T cell proliferation (Au-Yeung et al., 2010).

Defective Zap70 activation may also be influenced by differential expression of specific Src family members in naive and memory T cells. Herein, we confirm earlier observations that resting memory CD8<sup>+</sup> T cells express higher levels of p59 fyn than naive CD8<sup>+</sup> T cells (Kersh et al., 2003), which may explain why previous work has suggested that memory CD8<sup>+</sup> T cell recall expansion and effector functions can be p56 lck-independent in vivo (Tewari et al., 2006). In addition, we unexpectedly found increased expression of lyn kinase in central memory CD8<sup>+</sup> T cells, which may play a role in negative regulation of TCR signaling in memory T cells downstream of FcγRIIb (Starbeck-Miller et al., 2014). Despite expression of unique Src family kinase members in naive and central memory T cells, activation with a physiological stimulus induced similar levels of total Src family kinase activation. Comparable Src family activation was observed despite decreased levels of TCR on central memory versus naive T cells, suggesting that the efficiency of TCR signaling in memory T cells may be augmented. This finding may indirectly support previous studies suggesting that memory T cells express TCR oligomers that facilitate more efficient T cell activation measured by CD69 expression (Kumar et al., 2011). However, recent studies have also found that human naive and memory T cells differentially activate ERK, p38, and calcium flux upon anti-CD3 stimulation (Adachi and Davis, 2011). Thus, further studies on TCR signaling in primary cells are warranted to fully elucidate the distinct mechanisms used by naive and memory T cells.

The weak proliferative recall of memory T cells that we observed in three different model systems contrasts with the augmented effector functions that memory CD8<sup>+</sup> T cells are known to exhibit upon restimulation (Slifka and Whitton, 2001). Intriguingly, this implies that cytokine secretion and recall proliferation may require activation of distinct signaling pathways in memory T cells. Indeed, recent work has indicated that proliferation of CD4<sup>+</sup> T cells required stronger CD3 phosphorylation and subsequent Vav1–Notch1 association with the TCR than did CD69 expression or cytokine production (Guy et al., 2013). It is attractive to speculate that differential Src family kinase expression may control specific aspects of memory CD8<sup>+</sup> T cell functions. Indeed, recent evidence suggests that fyn activation regulates cytokine secretion but not cytotoxicity in NK cells via ADAP1-dependent stimulation of the Carma1–Bcl-10–MAP3K7 signalosome (Rajasekaran et al., 2013). Future studies are necessary to identify the localization and role of specific Src family members in stimulating specific memory T cell functions.

Our data indicates that Myc induction is reduced in memory as compared with naive T cells stimulated under the same conditions. Previous work has shown that T cell metabolic reprogramming and proliferative responses are dependent on cMyc expression (Wang et al., 2011). Herein, we find

that cMyc induction is directly tied to antigen dose and strength of TCR stimulation. Although both mitogen and IL-2 signaling can induce cMyc (Grandori et al., 2000), we observed strong up-regulation of cMyc within an hour of strong TCR stimulation, suggesting direct TCR-dependent activation. These results support previous confocal work indicating that cMyc nuclear localization correlates with TCR ligand avidity (Guy et al., 2013). Together, these data add to the growing number of transcription factors involved in translation of TCR signal strength into effector T cell differentiation programs (Moran et al., 2011; Man et al., 2013).

Intriguingly, we found that central memory CD8<sup>+</sup> T cells also expressed increased levels of multiple nonreceptor PTPs known to negatively regulate TCR activation. Of these, PTPN2 (TC-PTP) and PTPN22 (PEP/Lyp) have been shown to attenuate Lck and Zap70 signaling and set the threshold for T cell activation (Hasegawa et al., 2004; Wiede et al., 2011). In contrast, PTPN12 (PTP-PEST) positively regulates T cell expansion by modulating T cell–T cell homocjugate formation (Davidson et al., 2010). This class of PTPs may have a critical role in regulating TCR signal transduction, specifically in memory T cells, as mice deficient in either PTPN12 or PTPN22 exhibit altered T cell accumulation only upon secondary stimulation (Hasegawa et al., 2004; Davidson et al., 2010). At low doses, where Zap70 and Src family activation is limited, increased expression of these nonreceptor phosphatases likely reduces the strength of TCR signal that memory T cells perceive. It is interesting to speculate that the SNPs in PTPN2 and PTPN22 previously associated with autoimmune progression in type I diabetes, rheumatoid arthritis, and Crohn's disease (Todd et al., 2007; Wellcome Trust Case Control Consortium, 2007; Smyth et al., 2008; Espino-Paisan et al., 2011; Latiano et al., 2011; Hinks et al., 2012) may have the strongest impact on TCR signaling in memory T cells. Indeed, recent analysis of PTPN22–R619W knockin mice indicated that proliferation in response to anti-CD3 stimulation was differentially impacted in naive versus CD44<sup>hi</sup> memory CD4<sup>+</sup> T cells (Dai et al., 2013). Differences in phosphatase activity may also result from CSK-mediated recruitment of PTPN22 to lipid rafts (Vang et al., 2012), which are enriched in memory T cells. Further work defining localization-dependent activity of PTPN22 in naive and memory T cells is necessary to elucidate the mechanisms of genetically associated autoimmune diseases.

As memory T cells exist at an increased precursor frequency and exhibit greater responsiveness to common gamma chain cytokines than naive cells, more stringent control of their proliferative activity may safeguard against excessive and harmful reactions to cross-reactive self-antigen or a minor re-appearance of foreign antigen at an immune-privileged site. Indeed, our data shows that memory CD8<sup>+</sup> T cells specific for a pancreatic self-antigen proliferate less vigorously than naive cells. As T cells rapidly induce effector phenotypes upon stimulation, including specific chemokine receptor and integrin expression, it may be advantageous to more tightly restrict the proliferative capacity of memory T cells, not only to reduce

cytotoxic effector differentiation but also CD8<sup>+</sup> T cell accumulation in tissues sensitive to cytolytic damage. Stringent regulation of memory T cell proliferation does not imply that these cells lack the ability to produce cytokines. Indeed, recent work has suggested that although T cells do not initiate proliferation for several days after viral challenge, memory T cells secrete cytokines within only a few hours of infection (Whitmire et al., 2008). Our experiments do not address the antigenic thresholds required for cytokine production or cytolytic functions. Further studies defining the signal thresholds and cascades required to drive specific memory effector responses will likely inform our understanding of memory CD8<sup>+</sup> T cell function.

## METHODS AND MATERIALS

**Mice.** C57BL/6J and B6.SJL-PtprcaPep3b/BoyJ (CD45.1) mice (The Jackson Laboratory) were housed in specific pathogen-free conditions in the animal facilities at the University of Washington. Previously described OT-I TCR transgenic mice congenic for CD45.1 or CD45.1/CD45.2, and RIP-mOVA transgenic (Kurts et al., 1996) mice were bred and maintained in the same facilities. All experiments were done in accordance with the Institutional Animal Care and Use Committee guidelines of the University of Washington.

**Immunizations.** Mice were immunized with endotoxin-free OVA (Hyplos) or immune complexes containing Ova (IC-OVA) as previously described (den Haan and Bevan, 2002). In brief, rabbit anti-OVA IgG (MP Biomedicals) was purified over Protein G (Thermo Fisher Scientific), and chicken ovalbumin protein (OVA; Sigma-Aldrich) was reconstituted in PBS and dialyzed to remove free peptide. OVA was diluted in sterile PBS for direct injection or mixed with anti-OVA antibody and incubated for 15 min at 37°C. Mice were immunized with between 400 and 1.5 µg OVA alone or 150 µg OVA + 300 µg anti-OVA by tail vein injection.

**Naive and memory T cell adoptive transfer.** Naive CD45.1<sup>+</sup>CD45.2<sup>+</sup> OT-I T cells were purified from pooled spleen and lymph nodes using a MACS CD8 enrichment kit (Miltenyi Biotec) supplemented with anti-CD44-biotin (BD). Central memory CD45.1<sup>+</sup> OT-1 cells were generated either by adoptive transfer of in vitro activated OT-1 cells into B6 hosts as described previously (Wakim and Bevan, 2011) or by infection with VSV-OVA of B6 mice that had received 10<sup>6</sup> naive CD45.1 OT-1 T cells. At least 60 d after infection, memory OT-1 cells with >90% CD44<sup>hi</sup>CD62L<sup>hi</sup> phenotype were isolated from pooled spleen and lymph nodes by depletion of CD45.2<sup>+</sup> and non-CD8<sup>+</sup> cells using a MACS CD8 enrichment kit supplemented with anti-CD45.2-biotin. Isolated naive and memory OT-1 T cells were labeled with 2 µM CFSE and at least 10<sup>6</sup> cells injected at various times after immunization.

**Ex vivo IL-2 culture.** Single cell suspensions were prepared from spleen and lymph nodes of mice 18 h after adoptive transfer of CFSE-labeled naive and memory OT-1 T cells. Cell suspensions were cultured in the presence of 20 U/ml recombinant human IL-2 (rhIL-2; National Institutes of Health) for a further 48 h in RPMI 1640 containing 10% FBS, 10 mM Hepes, sodium pyruvate, MEAA, 50 µM 2-ME, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. The number of proliferating congenic OT-1 cells diluting CFSE was quantified by flow cytometry.

**DC subset presentation.** DCs were enriched and subsets sorted as previously described (Wakim and Bevan, 2011). In brief, spleen and lymph nodes were enzymatically digested for 30 min with continuous mechanical disruption in RPMI media containing 2% FCS, collagenase type 3 (Worthington), and 10 µg/ml Brefeldin A (Sigma-Aldrich). CD11c<sup>+</sup> cells were enriched by depletion of CD19<sup>+</sup>, Gr-1<sup>+</sup>, TER119<sup>+</sup>, and Thy1<sup>+</sup> cells, and CD11c<sup>+</sup>MHCII<sup>hi</sup> DC subsets were sorted into subsets on a FACSaria II. After a 30-min peptide

pulse, 2 × 10<sup>4</sup> sorted DCs were washed and co-cultured with either 5 × 10<sup>4</sup> CFSE-labeled congenic purified naive or memory OT-1 in U-bottom 96-well plates in duplicate. The number of proliferating OT-1 T cells as determined by CFSE dilution was quantified by flow cytometry after 60 h.

**Flow cytometry.** Direct comparison of intracellular protein expression was determined by adding purified congenic naive OT-1 T cells to bulk splenocytes containing an equal number of congenic memory OT-1 T cells with >90% CD44<sup>+</sup>CD62L<sup>+</sup> central memory phenotype. Cells were surface stained for Vα2 TCR, CD8α, and congenic marker, fixed with intracellular fixation solution (eBioscience), and permeabilized with perm wash (eBioscience). Nuclear expression was determined after permeabilization with FoxP3 staining kit (eBioscience). The following rabbit monoclonal antibodies were purchased from Cell Signaling Technology: Lck (clone 73A5), Lyn (clone C13F9), Zap70 (clone D1C10E), Syk (clone D115Q), SHP-1 (clone C15H6), p27 (clone D69C12), and cMyc (clone D84C12). Rabbit polyclonal anti-p15 was obtained from Cell Signaling Technology and goat polyclonal anti-PTPN2 was obtained from R&D Systems. Staining was developed using species-specific Fab'2-APC (Jackson ImmunoResearch Laboratories). Mouse monoclonal antibodies against PTPN12 (clone 4G6; Abnova), PTPN22 (clone 4F6; Abnova), p21 (clone SXM30; BD), Fyn (clone 1S; Abcam), and p18 (clone DCS118; Cell Signaling Technology) were directly conjugated to DyLight650 according to the manufacturer's protocols (Thermo Fisher Scientific).

Direct comparison of naive and memory TCR signaling was determined in vitro by addition of SIINFEKL peptide to cultures containing equal numbers of naive and memory OT-1 T cells in duplicate wells. After stimulation, cells were fixed with Fix Buffer I (BD), washed, surface stained for CD8 and congenic marker, permeabilized with Perm Buffer III (BD), and intracellularly stained with primary antibody. Rabbit monoclonal antibodies against SrcFamily pY419 (clone D49G4) and Rb pS780 (clone C84F6), and rabbit polyclonal antibody against Zap70 pY493 were obtained from Cell Signaling Technology. Staining was visualized with donkey anti-rabbit IgG Fab'2-APC (Jackson ImmunoResearch Laboratories). Data were recorded on a FACS-Canto II (BD) and analyzed using FlowJo software (Tree Star).

**Statistical analysis.** Mean fluorescence intensity and total numbers of divided OT1 were analyzed for statistical significance by unpaired two-tailed Student's *t* test using Prism (GraphPad Software).

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