

ORIGINAL ARTICLE

# IL-12 is required for mTOR regulation of memory CTLs during viral infection

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The induction of functional memory cytotoxic T lymphocytes (CTLs) is a major goal of vaccination against intracellular pathogens. Interleukin (IL)-12 is critical for the generation of memory CTLs, and inhibition of mammalian target of rapamycin (mTOR) by rapamycin can effectively enhance the memory CTL response. Yet, the role of IL-12 in mTOR's regulation of memory CTL is unknown. Here we hypothesized that the immunostimulatory effects of mTOR on memory CTLs requires IL-12 signaling. Our results revealed that rapamycin increased the generation of memory CTLs in vaccinia virus infection, and this enhancement was dependent upon the IL-12 signal. Furthermore, IL-12 receptor deficiency diminished the secondary expansion of rapamycin-regulated memory and resultant secondary memory CTLs were abolished. Rapamycin enhanced IL-12 signaling by upregulating IL-12 receptor  $\beta$ 2 expression and signal transducer and activator of transcription factor 4 phosphorylation in CTLs during early infection. In addition, rapamycin continually suppressed T-bet expression in both wild-type and IL-12 receptor knockout CTLs. These results indicate an essential role for IL-12 in the regulation of memory CTLs by mTOR and highlight the importance of considering the interplay between cytokines and adjuvants during vaccine design.

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## INTRODUCTION

Enhancement of memory cytotoxic T lymphocytes (CTLs) holds promise for vaccination against chronic viral infections, such as HIV. The generation of functional memory CTLs requires inflammatory cytokines along with antigen and co-stimulation.<sup>1–5</sup> Among the cytokines, interleukin (IL)-12 and type I interferon (IFN) have been identified as the major components for providing the third signal to induce fully functional memory CTLs.<sup>3,6–8</sup> The memory CTL response is compromised when CTLs, through receptor deficiencies, are unresponsive to these third-signal cytokines, as has been demonstrated in vaccinia virus (VV) and *Listeria monocytogenes* (LM) infections.<sup>9</sup> IL-12, in conjunction with antigen and co-stimulation, is capable of programming memory CTLs *in vitro*,<sup>9–11</sup> further supporting the pivotal role of IL-12 in memory CTL induction. IL-12 has been used in preclinical studies, yielding promising results. IL-12 enhances T helper type 1 and CTL responses when co-administered with antigens in gene transfer<sup>12</sup> induces functional memory CTLs when co-administered subcutaneously with peptide<sup>13,14</sup> and suppresses tumor growth.<sup>15–17</sup> Therefore, IL-12 is a critical stimulator of memory CTLs.

Mammalian target of rapamycin (mTOR) is a conserved signaling integrator for many environmental components, such as amino acids and growth factors.<sup>18</sup> Interestingly, mTOR was recently found to be a critical regulator of immune functions,<sup>19</sup> such as immune homeostasis,<sup>18</sup> activation,<sup>20,21</sup> differentiation<sup>22,23</sup> metabolism and migration.<sup>24,25</sup> Inhibiting mTOR via rapamycin enhances memory CTLs during lymphocytic choriomeningitis virus (LCMV)<sup>26</sup> and LM infections.<sup>27</sup> Although rapamycin directly interacts with IL-12 *in vitro* to regulate the balance of T-bet/Eomes expression,<sup>11</sup> it is unclear whether rapamycin's immunomodulatory effects require inflammatory cytokines during infection in animals.

Using adoptive transfer and receptor deficiency in a mouse model, we show that rapamycin substantially increased the quantity of functional and protective memory CTLs during VV infection. This rapamycin-induced regulation requires IL-12, as the absence of IL-12 signal reduced the memory CTL response. Additionally, rapamycin directly enhanced IL-12 signaling by upregulating signal transducer and activator of transcription factor 4 (STAT4) phosphorylation and consistently inhibited T-bet expression in both wild-type (WT) and IL-12 receptor deficient (IL-12RKO) CTLs in infected animals. More importantly, secondary memory CTLs were abolished when the IL-12 signal was absent. Taken together, these data indicate that IL-12 is essential for rapamycin regulation. Therefore, specific inflammatory cytokines may be necessary when rapamycin is used as an adjuvant.

## RESULTS

### Rapamycin enhances memory CTLs during VV infection

Administration of rapamycin to mice can promote memory CTLs in both LCMV<sup>26</sup> and LM infections.<sup>27</sup> We sought to understand whether rapamycin had similar effects on memory differentiation in VV infection. Purified naive OT-I CD8 T cells were transferred into naive B6 mice, and the recipients were infected with recombinant VV containing a chicken ovalbumin peptide (VV-OVA).<sup>28</sup> We previously found that high doses of rapamycin have a better regulatory function on IL-12-driven memory CTL programming *in vitro* than do low doses.<sup>10</sup> In addition, high doses of rapamycin can accelerate the transition of effectors to memory CTLs in LCMV infection.<sup>26</sup> We speculated that daily administration of high doses of rapamycin early in infection would be immunostimulatory, as this period corresponds to memory CTL

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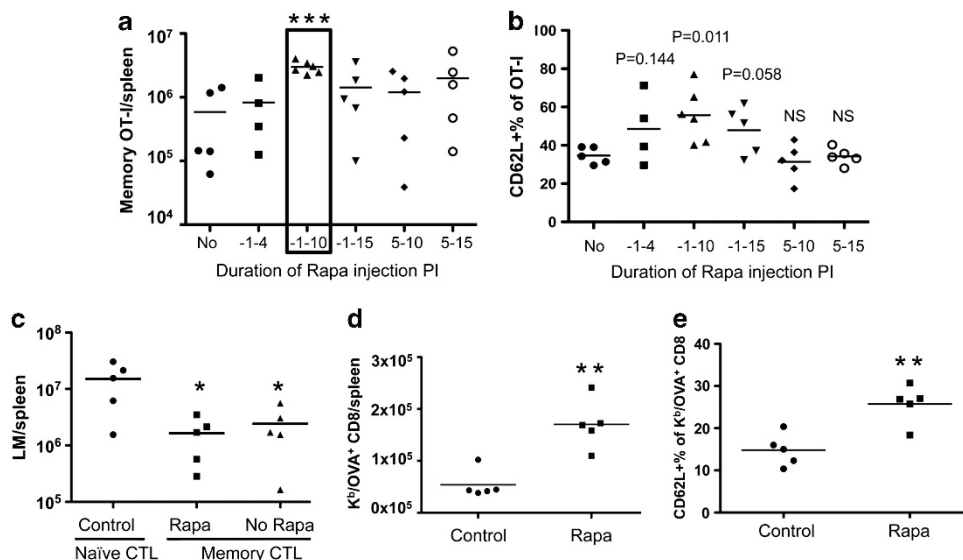
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programming by IL-12 *in vitro*.<sup>10</sup> A high dose of rapamycin was injected daily intraperitoneally during different time windows based on a pilot experiment revealing no difference between D10 and D30 for daily administration (Supplementary Figure S1). Memory OT-I cells were examined at D30 postinfection (PI). Consistent with the report by Araki *et al.*,<sup>26</sup> inhibition of mTOR by rapamycin significantly enhanced memory CTLs during VV infection by fourfold when administered from D-1 to D10 PI (Figure 1a and Supplementary Figure S2). The first injection window (D-1 to D4 PI) was not sufficient for rapamycin regulation, and continuous administration of rapamycin after D10 was not beneficial (Figure 1a). Thus, we used D-1 to D10 PI as the standard time window for rapamycin injection for the rest of this project, unless otherwise indicated. The immunostimulatory effect of rapamycin was not a consequence of VV infection delay by rapamycin, as VV was not detectable in tissues (spleen, lymph node (LN), peritoneal cavity) 5 days PI in both rapamycin-treated and untreated mice (data not shown). In LCMV infection, low doses of rapamycin applied during the expansion phase increased the frequency of memory CTLs, whereas high doses applied during the contraction phase accelerated memory differentiation.<sup>26</sup> Our data showed that administration of high-dose rapamycin during the early infection increased memory CTLs. The high dose did not change the kinetics of CTLs response but delayed both the expansion and contraction phases. The memory CTLs stabilized at a time (D30) comparable to the no rapamycin controls, consistent with an accelerated memory differentiation driven by high-dose rapamycin.<sup>26</sup> Similar to LCMV infection,<sup>26</sup> rapamycin upregulated CD62L expression in memory CTLs (Figure 1b). In addition, bulk splenocytes containing an equal number of memory OT-I cells ( $10^5$ ) were transferred into naive B6 mice. They were challenged the next day with recombinant LM containing chicken ovalbumin (LM-OVA) intravenously as we previously reported.<sup>9,29</sup> Memory OT-I cells generated with and without rapamycin achieved similar protection (Figure 1c).

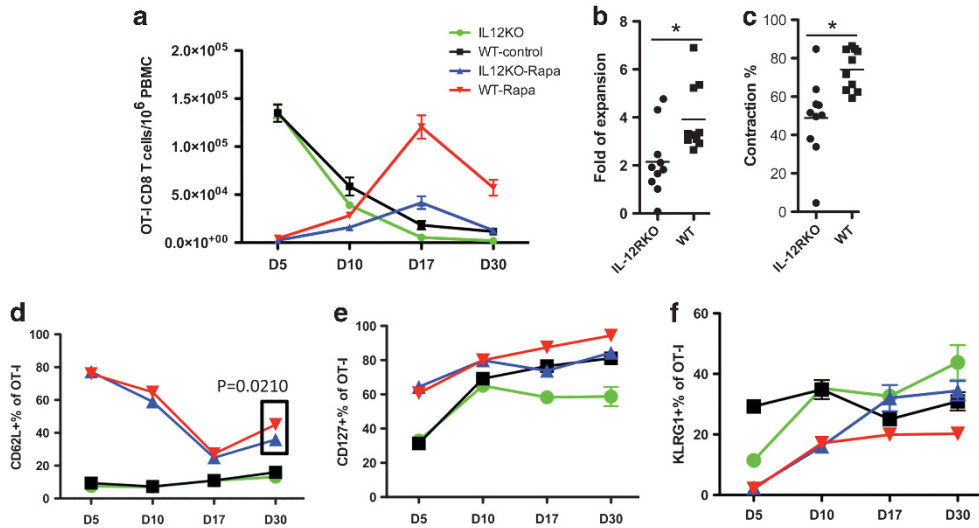
To further confirm the effects of rapamycin on the endogenous memory CTL response to VV-OVA infection, we infected naive B6 mice (no transfer) with VV-OVA with and without rapamycin treatment. K<sup>b</sup>/OVA tetramer was used to detect endogenous OVA-specific CD8 T cells.<sup>28</sup> We confirmed that rapamycin promoted endogenous memory CTLs similar to memory OT-I cells (Figure 1d). CD62L was upregulated in the rapamycin-treated endogenous memory K<sup>b</sup>/OVA-positive CTLs (Figure 1e). These data from both the transgenic system and the endogenous CTL response suggest that rapamycin increases the quantity of memory CTLs in response to VV infection and promotes a more central memory phenotype.

#### IL-12 increases CTL expansion following rapamycin treatment

To understand whether IL-12 signaling was required for rapamycin's regulation of memory CTL formation, OT-I cells of WT or IL-12RKO mice<sup>9</sup> (Supplementary Figure S3) were transferred into naive B6 recipients, which were infected with VV-OVA the next day. The recipient mice received daily rapamycin injections from D-1 to D10 PI as illustrated in Figure 1. Compared with untreated controls, effector CTL expansion in the rapamycin-treated WT and IL-12RKO groups was reduced by >10 times at the peak of expansion (D5) (Figure 2a). This is consistent with the report that a high dose of rapamycin inhibits expansion of effectors in LCMV infection.<sup>26</sup> However, CTLs significantly expanded between D5 and D10 in the rapamycin-treated WT and IL-12RKO groups (Figure 2a), and this expansion accelerated upon withdrawal of rapamycin until day 17. Notably, WT OT-I cells expanded almost two times more than IL-12RKO OT-I cells (Figure 2b) and supports the critical role of IL-12 in CTL expansion after rapamycin treatment. Interestingly, we noticed similar inhibition of rapamycin on CTL expansion *in vitro* but observed accelerated CTL expansion following transfer into recipients.<sup>10</sup> After D17, the CTL population contracted, and a fraction of expanded cells



**Figure 1.** Rapamycin enhances memory CTLs during VV infection. Purified naive OT-I cells were transferred into naive B6 recipients, which were infected with VV-OVA the next day. Rapamycin was injected daily at  $600 \mu\text{g kg}^{-1}$  through intraperitoneal at the time windows indicated in panel (a). (a) Memory OT-I cells in the spleens 30 days PI. (b) CD62L expression in memory OT-I cells from panel (a). (c) Splenocytes containing  $10^5$  memory OT-I cells were transferred into naive B6, which were challenged with LM-OVA the next day. Bacteria were cultured and counted 3 days after LM-OVA challenge in spleens. (d) Endogenous K<sup>b</sup>/OVA<sup>+</sup> memory CD8 cells in VV-OVA infected mice (without transfer of OT-I). Naive B6 mice (without transfer) were infected with VV-OVA, which were treated with or without rapamycin. (e) CD62L expression in K<sup>b</sup>/OVA<sup>+</sup> memory CD8 cells from panel (d). Rapamycin injection occurred daily from D-1 to D10 PI in panels (d and e). Student's *t*-test was performed comparing each of the groups with no rapamycin controls (a, b, d and e) or with naive CTL transferred controls (c). \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ , which will be the same in the rest of this study. The data are representative of three independent experiments with similar results.



**Figure 2.** IL-12 increases CTL expansion after rapamycin treatment. OT-I cells were purified from WT or IL-12RKO OT-I mice, which were transferred into naive B6 mice at  $10^7$ /mouse through tail vein. Recipients were infected with VV-OVA the next day. Daily rapamycin injection occurred from D – 1 to D10 PI. **(a)** Comparison of OT-I percentage of peripheral blood mononuclear cells in blood in different groups. Data were expressed as mean + s.e.m. of 6–10 mice for each group. **(b)** Comparison of expansion of OT-I after rapamycin withdrawal. Data were calculated by dividing the OT-I percentage at D17 by that at D10 (the last day for rapamycin injection). **(c)** Comparison of contraction of OT-I after rapamycin withdrawal. Data were calculated by dividing the OT-I percentage at D30 by that at D17. **(d–f)** Comparison of expression of CD62L, CD127 and KLRG1 in OT-I cells in blood samples from panel **(a)**. Data are representative of three experiments with similar results. Two-way ANOVA was performed in panels **(a, d, e and f)**. Student's *t*-test was performed in panels **(b and c)** and part of panel **(d)** as the square indicates.

became memory CTLs at D30, remaining stable thereafter (Figure 2a and data not shown). WT OT-I cells contracted more than IL-12RKO OT-I cells, based on lower expansion of IL-12RKO (Figure 2c). Therefore, IL-12 is critical for optimal CTL expansion and memory formation after rapamycin treatment.

Rapamycin treatment postponed the downregulation of CD62L until D10 (Figure 2d), which is consistent with its effects during *in vitro* stimulation.<sup>10</sup> The continued expansion of OT-I cells upon the withdrawal of rapamycin led to a quick downregulation of CD62L, although expression of CD62L remained higher than in their untreated counterparts (Figure 2d). CD62L was upregulated in rapamycin-regulated memory CTLs regardless of the presence or absence of IL-12 at D30 after the viral infection ( $P < 0.001$ , two-way analysis of variance (ANOVA)). However, there was a significant difference between WT and IL-12RKO OT-I cells treated with rapamycin—WT OT-I cells with rapamycin had slightly but significantly ( $P = 0.021$ , *t*-test) higher expression of CD62L than IL-12RKO treated with rapamycin. This suggests that IL-12 may partially contribute to the development of a more central memory phenotype (Figure 2d). Furthermore, IL-7 receptor  $\alpha$  (CD127) expression was upregulated by rapamycin in both groups ( $P < 0.001$ , two-way ANOVA), and WT OT-I cells expressed higher levels than IL-12RKO at D17 and D30 (Figure 2e). In addition, KLRG1 expression was downregulated by rapamycin ( $P < 0.001$  two-way ANOVA), but the absence of the IL-12 signal led to differential expression levels ( $P < 0.001$  two-way ANOVA) (Figure 2f). These data suggest that rapamycin favors a central memory CTL phenotype (CD62L<sup>hi</sup>/CD127<sup>hi</sup>/KLRG1<sup>lo</sup>), and the IL-12 signal may contribute to this phenotype.

#### Rapamycin enhances memory CTLs in tissues

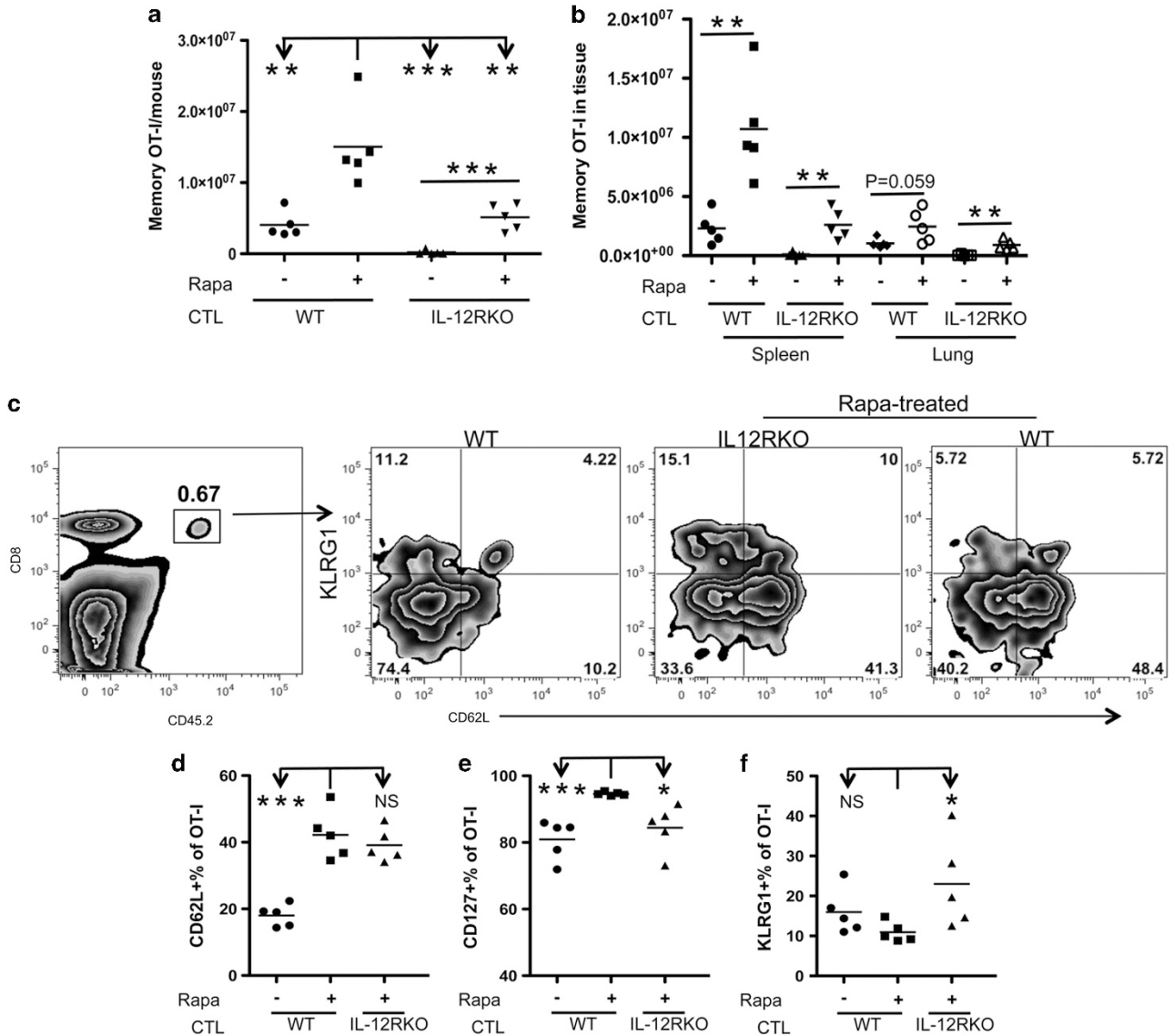
We sought to determine whether our observations regarding memory CTLs in blood also applied to CTLs in tissues. Memory mice, 40 days after VV-OVA infection and 30 days after rapamycin administration, were analyzed. Single cells were isolated from the peripheral LNs, spleen, bone marrow (two sets of femur) and lung. Similar to CTLs from the blood, rapamycin treatment significantly increased WT and IL-12RKO OT-I cells in tissues compared with

corresponding controls (Figure 3a). Yet, achieving optimal CTL memory requires IL-12: the IL-12 signal (WT) enhanced the rapamycin-treated memory threefold compared with IL-12 deficiency (rapamycin-treated IL-12RKO) (Figure 3a).

To investigate whether rapamycin altered migration of memory CTLs, we analyzed the tissue distribution of memory OT-I cells. Although rapamycin treatment increased the number of memory OT-I cells in tissues in both WT and IL-12RKO (Figure 3b), rapamycin-regulated memory OT-I cells tended to remain in the spleen ( $P = 0.057$ ) compared with CTLs not treated with rapamycin (Supplementary Figure S4A). This trend disappeared in IL-12RKO OT-I cells ( $P = 0.578$ ), which were retained in the spleen at similar percentages regardless of the exposure to rapamycin (Supplementary Figures S4A and B). In contrast, memory CTLs in the lung were significantly reduced (by about 10%) after rapamycin treatment in both the WT and IL-12RKO OT-I groups (Supplementary Figure S4A), consistent with the observation of enhanced central memory phenotype due to rapamycin. The memory OT-I cells in the spleens from rapamycin-treated mice exhibited increased expression of CD62L when compared with WT controls (Figures 3c and d). Similar to blood samples (Figures 2e and f), rapamycin-treated WT memory CTLs in the spleens had slightly but significantly higher expression of CD127 but lower expression of KLRG1 compared with their IL-12RKO counterparts (Figures 3e and f). These observations were similarly reflected in memory OT-I cells from most tissues (some differences were not significant), although the expression levels varied among tissues in the same animals (Supplementary Figures S4C–E). For example, memory CTLs in the lung had the lowest CD62L expression but the highest KLRG1 expression, which is consistent with an effector memory phenotype (Supplementary Figures S4C–E). These results suggest a general trend: rapamycin promotes a central memory phenotype of CTLs in tissues and in the periphery.

Memory CTLs derived from rapamycin treatments in the absence of the IL-12 signal are functional

Quantitative measurements of memory CTLs do not necessarily reflect functionality, as demonstrated by exhausted CTLs in



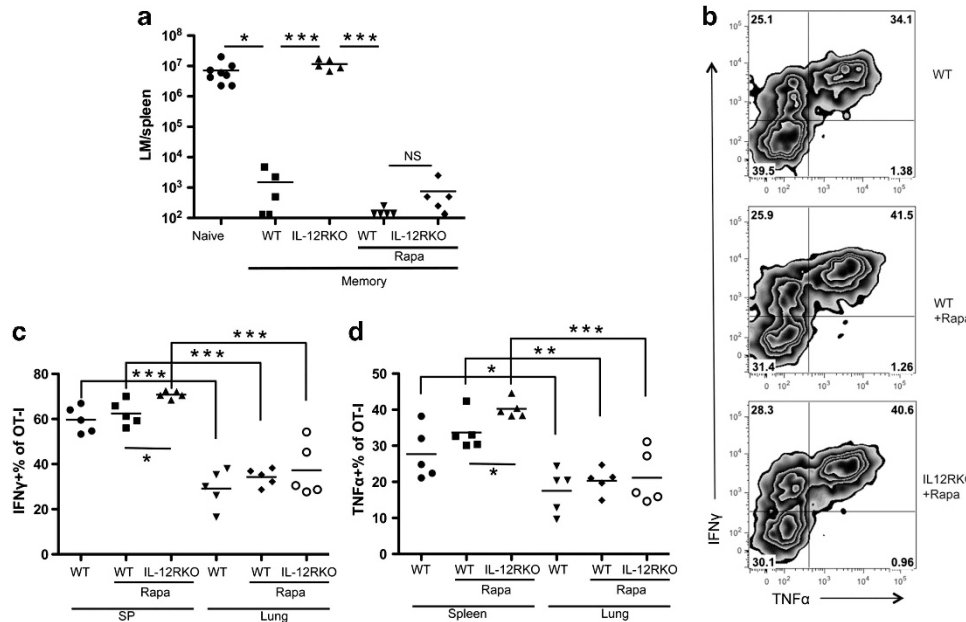
**Figure 3.** Rapamycin enhances memory CTLs in tissues. Memory OT-I cells were analyzed in memory mice (similar to those in Figure 2a) 40 days after VV-OVA infection. **(a)** Comparison of total memory OT-I cells from the peripheral LNs, spleen, lung and two sets of femur from each mouse. **(b)** Tissue distribution of memory OT-I cells in the spleen and lung. Data were calculated by dividing the number of memory OT-I in one tissue by the number in all the examined tissues. **(c)** Representative expression of CD62L/CD127/KLRG1 and corresponding statistics (Student's *t*-test) **(d–f)** of memory OT-I cells in the spleens from panel **(a)**. The experiment was repeated three times and similar results were obtained.

chronic LCMV infection.<sup>30–32</sup> To test whether the CTLs in this study were functional, memory mice were challenged with LM-OVA.<sup>9,10</sup> The memory mice that had originally received IL-12RKO OT-I were not protected against LM-OVA challenge, as is consistent with our previous report<sup>9</sup> (Figure 4a). Notably, treatment with rapamycin rescued functions of IL-12RKO CTLs and enabled them to respond to challenge, reaching levels of protection similar to WT with or without rapamycin treatments (Figure 4a). Endogenous K<sup>b</sup>/OVA CD8 T cells were undetectable (data not shown), suggesting that memory IL-12RKO OT-I were responsible for the enhanced memory protection in IL-12RKO OT-I transfer mice. IFN- $\gamma$  and tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) have been closely associated with memory CTL function, and these rapamycin-regulated memory IL-12RKO CTLs had slightly but significantly higher production of both molecules compared with WT controls (Figures 4b–d). Notably, there were significant differences in IFN- $\gamma$  and TNF $\alpha$  production by memory CTLs from different tissues

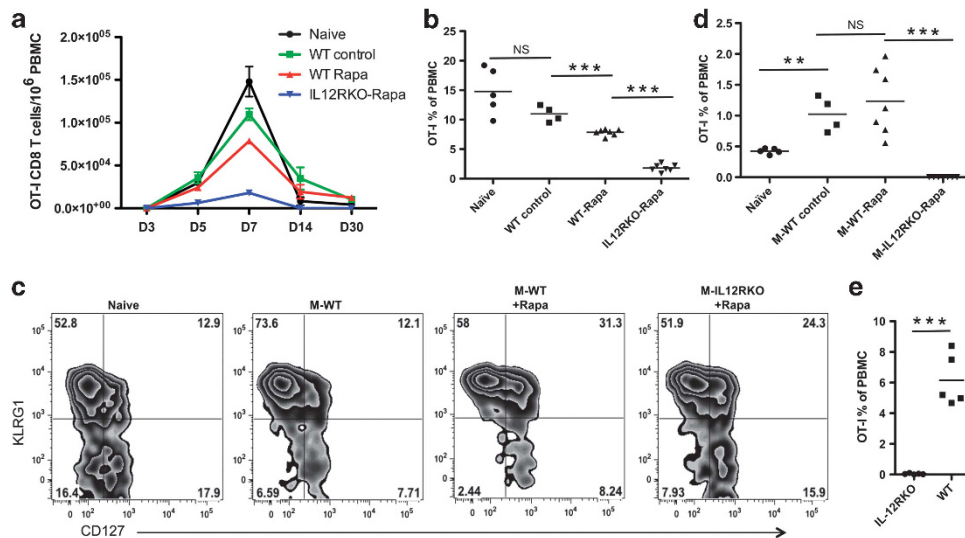
within the same individual: CTLs in the lungs produced the lowest amount of IFN- $\gamma$  and TNF $\alpha$ , whereas CTLs in the spleens, LNs and bone marrow produced more of these cytokines (Figures 4c and d and data not shown). These data suggest that the rapamycin-regulated memory CTLs are functional and protective, even in the absence of IL-12.

#### IL-12 is required for secondary expansion of memory CTLs regulated by rapamycin

A functional memory response is characterized by rapid expansion and quick control of reinfection upon pathogen re-challenge.<sup>33,34</sup> To test secondary expansion ability, an equal number ( $10^5$ ) of memory OT-I from each treatment group was transferred into naive recipients, which were then challenged with LM-OVA. OT-I became detectable at D5, peaked at D7 and contracted thereafter (Figure 5a). IL-12RKO OT-I had the smallest expansion at D7,



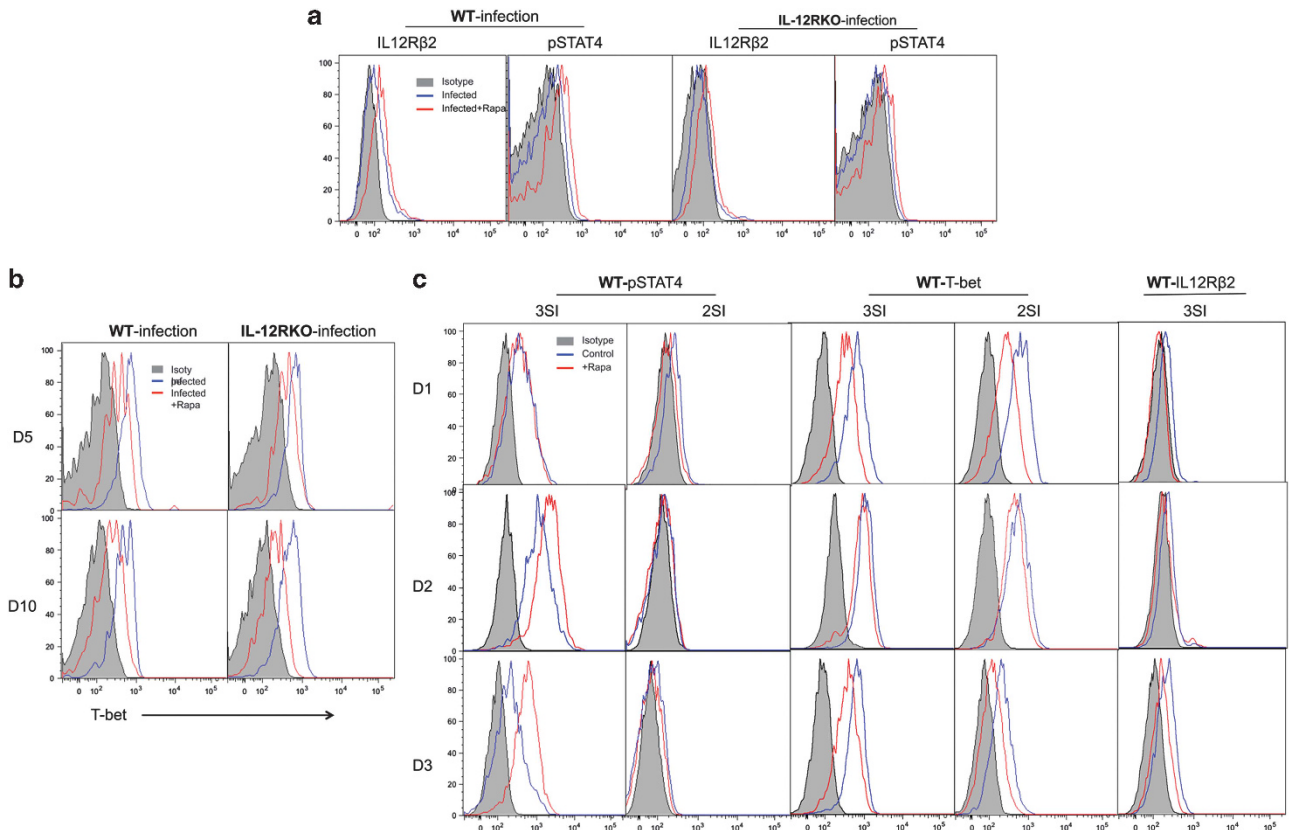
**Figure 4.** Rapamycin-regulated memory CTLs are functional in the absence of the IL-12 signal. (a) Memory mice (similar to those in Figure 2a) were challenged with LM-OVA, and bacterium was recovered from the spleen 3 days after challenge. (b–d) Resting memory OT-I cells in different tissues were examined for the production of IFN- $\gamma$  and TNF $\alpha$ . Representative cytokine expression in the spleen (b) and comparison between the spleen and lung (c and d). These are representative of three independent experiments with similar results.



**Figure 5.** IL-12 is required for secondary expansion of memory CTLs regulated by rapamycin. Naive mice having received naive or IL-12RKO OT-I cells were split into two groups: rapamycin-treated and untreated control. These mice were then infected with VV-OVA. Splenocytes containing 10<sup>5</sup> memory OT-I cells from each of the treatments were transferred into naive B6 mice, which were challenged the next day with LM-OVA. Memory IL-12RKO OT-I cells without rapamycin were at or below detectable level, hence were excluded in transfer. OT-I populations were tracked in the blood at various time points. (a) Kinetics of OT-I populations. Data are expressed as mean + s.e.m. of 4–7 mice. Comparison of OT-I percentage of peripheral blood mononuclear cells at D7 (b) or D30 (d) after LM-OVA challenge. (c) Comparison of the expression of KLRG1/CD127/CD62L in OT-I cells at D7 after LM-OVA challenge. (e) Mice that have received rapamycin-treated first memory OT-I cells (IL-12RKO and WT) were infected with LM-OVA as carried out in panel (a). These memory mice were challenged again with VV-OVA 60 days after LM-OVA infection, and CTL expansion was examined on D5. The results are representative of two separate experiments with similar results. Student's *t*-test was performed in panels (b, d and e).

which was significantly lower than the other groups (Figure 5b). Furthermore, this group (IL-12RKO) contracted the most, becoming almost undetectable at D14 postchallenge (Figure 5a). Interestingly, rapamycin-regulated WT memory OT-I cells were significantly lower than WT memory controls at D7 (Figure 5b), but both achieved a similar level of secondary memory (D30 after

re-challenge Figure 5a). Additionally, the absence of IL-12 signaling in the primary response caused weaker activation of memory CTLs, as demonstrated by a lower KLRG1 expression and reduced downregulation of CD62L at D7 (Figure 5c and Supplementary Figures S5A–C) and D5 (data not shown). The extent of expansion was predictive of the resultant secondary



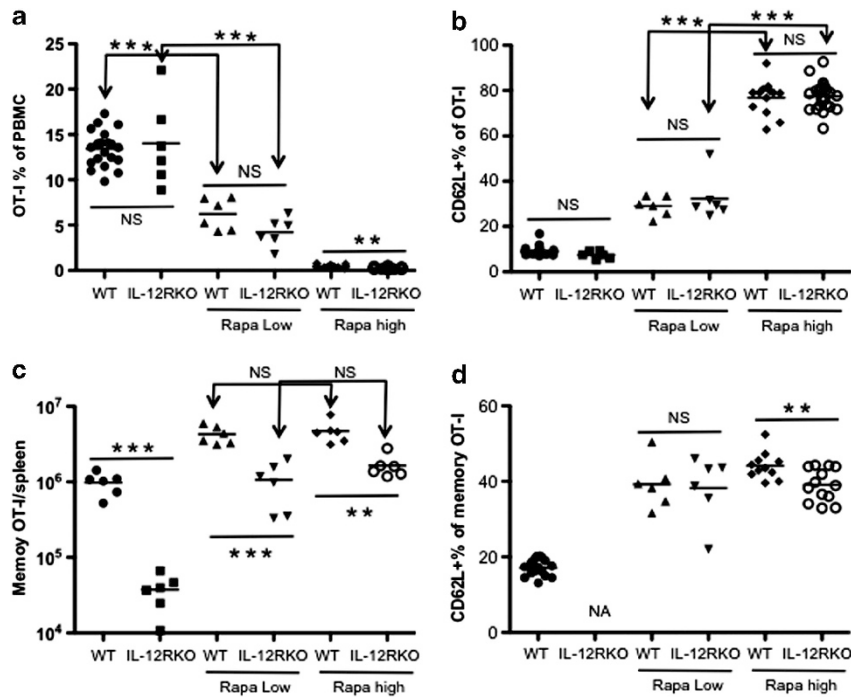
**Figure 6.** Rapamycin enhances IL-12 signaling in early infection and consistently inhibits T-bet expression. Naive WT or IL-12RKO OT-I cells were transferred into recipient B6 mice, which were infected with VV-OVA the next day. High doses of rapamycin were administered daily between D – 1 and D10 after VV-OVA infection. OT-I cells in the spleens were examined at days 5 (**a**) and 10 after infection (**b**). The results are representative of five mice per group, and similar data were obtained in two separate experiments. (**c**) Sorted WT OT-I cells were stimulated with 3SI (antigen + B7 + IL-12) or 2SI (antigen + B7) in the presence or absence of rapamycin as we have previously reported.<sup>10</sup> Programmed CTLs were examined at day 3 poststimulation. The T-bet was examined on effector CTLs generated *in vivo* (**b**) and *in vitro* (**c**). These are representatives of two independent experiments with similar results.

memory: secondary memory CTLs were undetectable in the IL-12RKO + rapamycin group (Figure 5d). Secondary memory from either WT memory or WT + rapamycin memory CTLs was higher than in naive controls (Figure 5d). To confirm the absence of memory CTLs, memory mice in the IL-12RKO + rapamycin group and WT + rapamycin group were challenged with VV-OVA at D60 after LM-OVA infection. There was no detectable expansion of OT-I at D5 in the IL-12RKO + rapamycin group, whereas a huge expansion was detected in WT (Figure 5e). Collectively, lack of the IL-12 signal causes defective secondary expansion and abolishes secondary memory formation.

#### Rapamycin enhances IL-12 signaling in early infection and consistently inhibits T-bet expression

Rapamycin's enhancement of memory CTL formation may be due to direct interactions with IL-12 signaling within CTLs or result indirectly from interactions with other cells. To address this question, naive WT and IL-12RKO OT-I cells were transferred into recipient B6 mice, which were infected with VV the next day. Rapamycin was administered as in Figure 2. OT-I cells were analyzed for IL-12 signaling and other pathways at different time points PI. IL-12 receptors are composed of two subunits,  $\beta 1$  (shared with IL-23) and  $\beta 2$  (binding p35 of IL-12, so is unique to IL-12).<sup>35–37</sup> Our IL-12RKO OT-I cells are deficient in the  $\beta 1$  subunit.  $\beta 1$  and  $\beta 2$  are differentially expressed in immune cells.<sup>38</sup> In naive CD4 cells,  $\beta 1$  is expressed but  $\beta 2$  is absent.<sup>39</sup> The expression of  $\beta 2$  is induced by IFN- $\gamma$  but inhibited by IL-4 during activation.<sup>39</sup> In CD8 T cells, both  $\beta 1$  and  $\beta 2$  can be regulated by cytokine stimulation (IL-12 or type I

IFN), but the speed and magnitude of upregulation is different between the two subunits. The transcriptional expression of  $\beta 2$  was upregulated earlier and with greater magnitude than was  $\beta 1$ .<sup>40</sup> Administration of rapamycin increased IL-12R  $\beta 2$  expression in both WT and IL-12RKO OT-I cells during early infection (days 3–5) but not  $\beta 1$  (Figure 6a and data not shown). Type I IFN receptor subunit 1 was not affected by rapamycin (Supplementary Figure S6A). This indicates that IL-12R  $\beta 2$  is upregulated by rapamycin. In addition to the receptor expression, rapamycin upregulated the phosphorylation of STAT4 in both WT and IL-12RKO OT-I cells but not the expression of Janus-activated kinase 2 on the protein level (Figure 6a). This suggests that rapamycin directly enhances STAT4 activation during early infection through the IL-12 signaling pathway and/or other cytokines.<sup>9,41–43</sup> T-bet is a transcription factor responsible for CTL effector function.<sup>44,45</sup> Rapamycin regulates IL-12-driven memory programming by inhibiting T-bet and promoting Eomes expression.<sup>11</sup> Consistent with this, administration of rapamycin suppressed T-bet expression in both WT and IL-12RKO CTLs at days 5 and 10 PI (Figure 6b and data not shown), but Eomes expression was not affected (Supplementary Figure S6B). Thus, rapamycin's suppression of CTL effector function may contribute to the enhanced memory in both WT and IL-12RKO OT-I cells. Interestingly, mTOR phosphorylation was not altered by rapamycin at days 5 and 10 PI, indicating that rapamycin may work through pathways other than mTOR (Supplementary Figure S6C). Therefore, our findings suggest that rapamycin can both directly augment IL-12 signaling during early infection and suppress CTL effector function.



**Figure 7.** Long-term administration of low doses of rapamycin enhances memory CTLs to levels comparable to high doses. Naive WT or IL-12RKO OT-I cells were transferred into recipient B6 mice, which were infected with VV-OVA the next day. Low doses of rapamycin were administered daily between D – 1 and D30 after VV-OVA infection, whereas high doses were administered between D – 1 and D10 PI. OT-I populations were tracked in blood samples. (a, c) Comparison of OT-I percentage of peripheral blood mononuclear cells at day 5 (a) or memory OT-I in the spleen at day 40 (c) after VV-OVA infection. (b, d) Comparison of expression of CD62L in OT-I in blood samples at D5 and D40 after VV-OVA infection. The results are representative of two separate experiments with similar results. Student's *t*-test was performed in panels (a–d).

To confirm the direct effects of rapamycin on IL-12 signaling observed in animals, sorted naive OT-I cells were cultured in the presence (3SI) or absence (2SI) of IL-12 in addition to antigen and B7 stimulation.<sup>10,46</sup> Indeed, rapamycin directly enhanced and extended STAT4 phosphorylation when IL-12 was present (Figure 6c). Consistent with the data in VV infection (Figure 6a), rapamycin directly inhibited T-bet expression independent of IL-12 (Figure 6c) as previously reported.<sup>11</sup> In contrast to *in vivo*, IL-12R $\beta$ 2 was inhibited by rapamycin in both 2SI and 3SI stimulation (data not shown). Therefore, rapamycin can directly enhance IL-12 signaling, but this does not necessarily occur through direct regulation of IL-12 receptors.

#### Long-term administration of rapamycin at low doses is equally effective as high doses

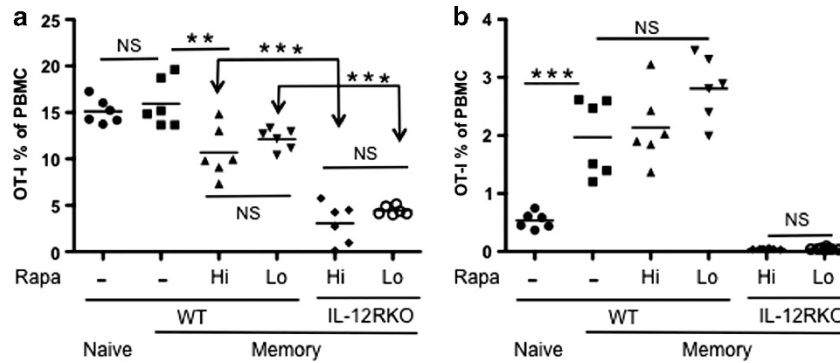
Long-term administration of low doses of rapamycin enhances memory CTLs in LCMV infection.<sup>26</sup> To test whether the same is true in VV infection, naive OT-I cells were transferred into B6 mice, which were infected with VV-OVA. Rapamycin was administered at either low doses from – 1 to 30 days PI or high doses from – 1 to 10 PI. Indeed, high doses of rapamycin (from – 1 to 10 PI) dramatically suppressed CTL expansion in both WT and IL-12RKO (Figure 7a). Yet, low doses of rapamycin inhibited CTL expansion in both WT and IL-12RKO CTLs, albeit more in IL-12RKO (Figure 7a). This suggests that inhibition of CTL expansion by rapamycin is dose-dependent, and the IL-12 signal may lessen this inhibition, at least partially.

With regards to surface molecules, there was no significant difference in KLRG1 and CD127 expression levels between both doses (data not shown). However, high expression of CD62L was associated with high doses of rapamycin (Figure 7b), whereas CD62L expression was dampened under low doses of rapamycin

in both WT and IL-12RKO. Interestingly, rapamycin's regulation of CD62L at the expansion stage is not dependent on IL-12 signaling (Figure 7b). Despite the differences in expansion and expression of surface molecules, memory CTLs reached similar levels in both WT and IL-12RKO OT-I regardless of the dose of rapamycin (Figure 7c). Furthermore, CTLs from both doses tended towards central memory phenotype—CD62L positive and mostly KLRG1 negative and CD127 positive (Figure 7d and data not shown). These data suggest that the long-term administration of low doses of rapamycin has similar effects on memory CTLs compared with short-term administration of high doses.

#### Requirement of the IL-12 signal for memory expansion is independent of the rapamycin dosage

It is possible that the impaired secondary expansion of rapamycin-regulated memory IL-12RKO CTLs is a consequence of high dosage. To address this question, spleen cells containing an equal number of memory OT-I from each treatment (high and low doses of rapamycin) were transferred into naive B6 recipients, which were challenged with LM-OVA the next day. At the peak of response (day 7 after re-challenge), rapamycin-regulated IL-12RKO OT-I were significantly lower than WT regardless of the dosage used during primary activation (Figure 8a), and expansion was only detectable 5 days after re-challenge (data not shown). Consistently, resultant secondary memory CTLs were abolished in IL-12RKO OT-I derived from both high and low doses of rapamycin (Figure 8b). No phenotypic difference was observed in resultant secondary memory CTLs from low and high dose rapamycin-regulated primary WT memory (data not shown). Therefore, the requirement of IL-12 for secondary memory expansion is independent of the rapamycin dosage.



**Figure 8.** Requirement of the IL-12 signal for memory expansion is independent of the rapamycin dosage. Naive mice, having received naive or IL-12RKO OT-I cells, were infected with VV-OVA with high or low doses of rapamycin. Splenocytes containing  $10^5$  memory OT-I cells from each of the treatments were transferred into naive B6 mice, which were challenged the next day with LM-OVA. Naive and WT memory controls (without rapamycin) were included. OT-I populations were tracked in blood samples at different time points. **(a)** Comparison of OT-I percentage of peripheral blood mononuclear cells in the blood at D7 **(a)** or at D30 **(b)** after LM-OVA challenge. The results are representative of two separate experiments with similar results. Student's *t* test was performed in panels **(a, b)**. One-way ANOVA was performed in panel **(b)** for comparison of three groups.

## DISCUSSION

Inhibiting mTOR by rapamycin effectively enhances memory CTLs in LCMV and *Listeria* infections.<sup>26,27</sup> Yet, whether the immunostimulatory effects of rapamycin require the presence of inflammatory cytokines is unknown. In this report, we confirmed that rapamycin enhances the formation of functional memory CTLs in VV infection and demonstrated that IL-12 signaling is necessary for achieving the optimal memory CTL response.

Consistent with our previous report,<sup>9</sup> IL-12 signal is required for memory formation. Deficiency of the IL-12 signal led to almost undetectable memory, despite similar effector expansion (Figure 2a). When rapamycin was administered to recipients, memory CTLs increased (Figure 2a). However, the presence of IL-12 signaling significantly enhanced the effects of rapamycin by 3–4 fold and shifted the CTL population to a more central memory phenotype. As IL-12 has a critical role in the differentiation of T helper type 1 and the establishment of a strong CTL response,<sup>47,48</sup> it is not surprising that this cytokine is required for optimal memory CTL formation following rapamycin treatment. Cessation of rapamycin treatment in primary VV-OVA infection enhanced effector expansion (Figure 2b) and subsequently improved memory CTL formation (Figure 2a). Consistent with a recent report from Ahmed *et al.*,<sup>26</sup> high doses of rapamycin inhibited effector expansion (Figure 2a). However, this strong inhibition did not abolish expansion—CTLs still expanded substantially when high doses of rapamycin were administered (Figure 2a). In addition, these effectors exhibited a period of delayed expansion upon termination of rapamycin treatment, and IL-12 contributed to the strength of this post-rapamycin expansion (Figure 2a). Compared with long-term administration of low doses of rapamycin, high doses yielded a similar effect within a shorter time window (Figure 7c).

Rapamycin promotes a central memory phenotype in a monkey model<sup>26</sup> and can program memory CTLs in short-term culture *in vitro* in the presence of IL-12.<sup>10,11</sup> In support of these findings, we found that rapamycin drove upregulation of CD62L regardless of the presence or absence of IL-12. However, the lack of the IL-12 signal reduced the expression of CD127 (IL-7 receptor alpha), which suggests decreased responsiveness to IL-7, a critical cytokine for the maintenance and homeostasis of memory CTLs.<sup>2,49–52</sup> Furthermore, the absence of IL-12 signal increased KLRG1 expression, an inhibitory receptor for T cells and a marker for short-lived effectors.<sup>53,54</sup> IL-12 marginally affected CD62L expression, if any (Figures 2d, 3d, 7b and Supplementary Figure S4C). These data indicate that memory CTL regulation by

rapamycin requires IL-12 to maintain a strong and healthy central memory CTL phenotype. This quantitative and qualitative regulation by rapamycin was similarly achieved from both high (Figure 2) and low doses (Figure 7). The requirement of IL-12 for the secondary memory response is evident. Rapamycin-regulated memory IL-12RKO CTLs expanded much less than WT CTLs treated with rapamycin. Moreover, there was no detectable secondary memory (Figures 5 and 8). As a common practice in vaccination, boosting with either vectors or adjuvant is used to increase the quantity and quality of memory CTLs.<sup>29,55–57</sup> Our data clearly suggest that enhancing memory CTLs using an mTOR inhibitor, such as rapamycin, requires IL-12 for both optimal primary memory and functional secondary responses. Of course, this does not necessarily exclude the need for other inflammatory cytokines, such as type I IFN, which are critical for the immune response against certain infectious pathogens, such as LCMV.<sup>34</sup>

Rapamycin may directly and indirectly regulate IL-12 signaling. IL-12R  $\beta$ 2 expression was enhanced by rapamycin, whereas no change was observed in  $\beta$ 1 expression during infection (Figure 6a). This could indicate that rapamycin affects IL-12 function in memory generation through differential regulation of IL-12 receptor subunits. However, both IL-12R $\beta$ 1 and  $\beta$ 2 were inhibited by rapamycin in CTLs when IL-12 was provided *in vitro* (Figure 6c). Therefore, the enhanced expression of IL-12R $\beta$ 2 by rapamycin during infection may be indirect, possibly occurring through other mechanisms. More importantly, inhibition of mTOR *in vitro* in the presence of IL-12 leads to enhanced memory programming,<sup>10,11</sup> suggesting that mTOR may affect downstream IL-12 signaling. Although the IL-12 signaling was disrupted in IL-12RKO OT-I cells due to  $\beta$ 1 deficiency, the STAT4 phosphorylation was similarly upregulated by rapamycin during the early infection (Figure 6a). In addition, rapamycin enhanced STAT4 phosphorylation in CTLs *in vitro* only in the presence of IL-12 (Figure 6c), suggesting that this may be due to the combined effects of IL-12 and other cytokines, such as type I IFN<sup>41–43,58</sup> and IL-3, IL-5 and IL-6.<sup>59–66</sup> Importantly, these effects were transient and only happened early in the infection, suggesting that the regulatory function of rapamycin for cytokine signaling may be generally short-lived. Rapamycin might also influence other components involved in IL-12 signaling that have not been addressed in this study. A global comparison of transcriptome or protein profiling between rapamycin-treated and control in both WT and IL-12RKO OT-I cells is currently underway and will provide more defined answers about the molecular mechanisms underlying rapamycin regulation.



It was recently reported that a third signal is required for secondary expansion of memory CTLs in a pathogen-dependent manner.<sup>34</sup> Different pathogens may cause distinct inflammatory milieus, and the induction of memory CTLs depends on unique cytokines, such as type I IFN for LCMV<sup>8</sup> and IL-12 for VV and LM.<sup>9,34</sup> The ability of CTLs to undergo secondary expansion requires the presence of pathogen-specific third-signal cytokines during priming.<sup>34</sup> Our data further support this discovery by illustrating that rapamycin-regulated memory CTL expansion requires a third signal during priming. We cannot rule out the possibility that IL-12 is required for the secondary expansion of memory CTLs, as in this experimental setting there is a lack of IL-12 signaling in both priming and memory stages. Once available, a conditional knockout model will be more suitable to address this question. Although the requirements for reactivating memory CTLs are still subject to debate, dendritic cells are essential for optimal CTL responses to secondary infections.<sup>67,68</sup> This implies that co-stimulation and/or inflammation is essentially involved in the reactivation of memory CTLs.<sup>69</sup> Recently, we reported that boosting with peptide requires adjuvant for memory CTL generation,<sup>29</sup> hence it appears that cytokines are needed. The immune response to live attenuated pathogens is usually stronger than that against killed vaccines.<sup>56,70</sup> Thus, induction of functional memory CTLs using killed vaccines is very challenging and often requires effective adjuvants and multiple boosts.<sup>13,14,56,70,71</sup> As shown in this report, the inhibition of mTOR and the provision of the IL-12 signal may provide the stimulation necessary to enhance the immune response against killed pathogens.

In summary, we found that IL-12 is critical for rapamycin regulation of memory CTLs in two aspects: (1) IL-12 enhances the regulatory function of rapamycin quantitatively and qualitatively. (2) The presence of IL-12 during priming is required for secondary expansion of memory CTLs regulated by rapamycin. When an mTOR inhibitor is used as an adjuvant to enhance memory CTLs during vaccination, it is important to provide sufficient required inflammatory cytokines, such as IL-12.

## MATERIALS AND METHODS

### Mice and reagents

OT-I mice and OT-I mice deficient for IL-12 receptor  $\beta 1$  (gifts from Dr Mescher, University of Minnesota) have a transgenic T-cell receptor specific for H-2K<sup>b</sup> and OVA<sub>257–264</sub>.<sup>9,10,72</sup> Mice were maintained under specific pathogen-free conditions at the University of Maryland, and these studies have been reviewed and approved by the Institutional Animal Care and Use Committee. C57BL/6 male mice were purchased from the National Cancer Institute (Frederick, MD, USA). VV preferentially accumulates in the ovaries,<sup>73,74</sup> which may cause variation in CTL activation under different treatments. As a result, only male recipient mice were used for VV infection. Phospho-Stat4 (Tyr693) (D2E4) and Janus-activated kinase 2 (D2E12) were purchased from Cell signaling Technology (Danvers, MA, USA). All the rest directly conjugated fluorescent antibodies were purchased from BD Biosciences (San Jose, CA, USA), eBioscience (San Diego, CA, USA) or Biolegend (San Diego, CA, USA). Rapamycin were purchased from EMD (Gibbstown, NJ, USA). K<sup>b</sup>/OVA tetramer is a gift from Dr Jameson from the University of Minnesota.

### Viruses and bacteria

Recombinant VV-GFP-JAW-OVA (VV-OVAp) expresses the OVA<sub>257–264</sub> epitope fused C-terminally to green fluorescent protein and the transmembrane region of JAW-1 (a gift from Dr Jameson, University of Minnesota).<sup>28,75</sup> Viral titers were determined by plaque assays using 143B cells, and mice were infected intraperitoneally with  $5 \times 10^6$  plaque-forming units. Recombinant LM (a gift from Dr Jameson, University of Minnesota) expressing full-length secreted ovalbumin (LM-OVA) was used for inoculation at either  $10^4$  CFU (colony-forming units) per mouse (for secondary expansion of memory CTLs) or  $5 \times 10^5$  CFU per mouse (for memory CTL protection) via intravenous. Mouse spleens were harvested 3 days after LM-OVA challenge, and LM-OVA was cultured using TSB plates for comparison of protection as in our previous reports.<sup>9,10</sup>

### Administration of rapamycin

Mice were injected daily with rapamycin (EMD) through intraperitoneal during a treatment period. Two different treatment periods were used: (1) high dose ( $600 \mu\text{g kg}^{-1}$ ) administration during VV-OVA infection (day  $-1$  before day 10 PI) or as indicated; and (2) low dose ( $75 \mu\text{g kg}^{-1}$ ) administration during VV-OVA infection (day  $-1$  before infection to day 30 PI), as previously reported.<sup>26</sup> Control mice received sham treatment.

### Naive T-cell purification

This was performed as previously reported.<sup>9,10</sup> Briefly, inguinal, axillary, brachial, cervical and mesenteric LNs were harvested from WT OT-I or IL-12RKO OT-I mice, pooled and disrupted to obtain a single-cell suspension. Cells were incubated with FITC (fluorescein isothiocyanate)-labeled antibodies specific for CD4, B220, I-A<sup>b</sup> and CD44. Anti-FITC magnetic MicroBeads (Miltenyi Biotech, Auburn, CA, USA) were then added, and the suspension was passed through separation columns attached to a magnetism-activated cell sorter magnet. Cells that did not bind were collected with a purity  $>95\%$  CD8<sup>+</sup> cells and  $<0.5\%$  CD44<sup>hi</sup> cells.

### Adoptive transfer and flow cytometric analysis

This is the same as we previously reported.<sup>9,10</sup> Purified OT-I cells were adoptively transferred into normal C57BL/6Ncr mice by intravenous (tail vein) injection at  $10^5$  cells per mouse, and OT-I cells were identified as CD8<sup>+</sup>CD45.2<sup>+</sup> cells. Blood samples were drawn at the indicated times, and the analysis of memory CTLs was based on samples from blood and/or tissues. Single-cell suspensions were prepared, viable cell counts were performed (trypan blue) and the percentage of OT-I cells in the sample was determined by flow cytometry. Background for determining the OT-I cell numbers was determined by identical staining of cells from normal C57BL/6 mice (no adoptive transfer). Analysis was done using a FACS Calibur flow cytometer and the CELLQuest software (BD Biosciences) to determine the percentage and total OT-I cells in the samples. Flowjo software (Tree Star Inc., Ashland, OR, USA) was used for data analysis.

### Tissue harvest and digestion

Mice were euthanized by CO<sub>2</sub>, and the peripheral LNs and spleens were directly picked up and homogenized using 15-ml glass grinders. Lungs were perfused using  $1 \times$  phosphate-buffered saline at about 30 ml per mouse, cut into small pieces ( $1 \text{ mm}^3$ ), homogenized with a 10-ml pipette and resuspended in 4 ml Collagenase D (Roche, Indianapolis, IN, USA). For complete digestion, lung tissues were kept in a water bath ( $37^\circ\text{C}$ ) for 25 min. Digestion was stopped by the addition of 0.1 M EDTA, and digested tissues were homogenized using glass grinders. Bone marrow was harvested by flushing cut bones with  $1 \times$  phosphate-buffered saline.

### Intracellular cytokine staining after *in vitro* stimulation

Single-cell suspension from adoptively transferred mice was incubated at  $2 \times 10^6$  cells  $\text{ml}^{-1}$  in RP-10 with  $0.2 \mu\text{M}$  OVA<sub>257–264</sub> peptide and  $1 \mu\text{l}$  Brefeldin A (Biolegend) for 3.5 h at  $37^\circ\text{C}$ .<sup>9,10</sup> Cells were fixed in fixing buffer (Biolegend) for 15 min at  $4^\circ\text{C}$ , permeabilized in Saponin-containing Perm/Wash buffer (Biolegend) for another 15 min at  $4^\circ\text{C}$  and stained with phycoerythrin-conjugated antibody to IFN- $\gamma$  or allophycocyanin-conjugated antibody to TNF $\alpha$  for 30 min at  $4^\circ\text{C}$ . Cells were then washed once with Perm/Wash buffer and once with phosphate-buffered saline containing 2% fetal bovine serum.

### Statistical analysis

Data was graphed and analyzed using a two-tailed Student's *t*-test or two-way ANOVA (GraphPad Prism 5.0 software; GraphPad Prism, La Jolla, CA, USA).<sup>9,10</sup> Comparisons with a *P*-value of  $<0.05$  were considered significantly different.

### CONFLICT OF INTEREST

The authors declare no conflict of interest.

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