ABSTRACT

Title of Document: **THE ROLE OF INTERLEUKIN-12 FOR MTOR REGULATION OF MEMORY CTLS.**

Karla Garcia, Master of Science, 2014

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A major goal of vaccines is to induce functional immune memory, and efforts to improve the efficacy of vaccines targeting memory CTLs have revealed an important immunoregulatory role of rapamycin, a specific mTOR inhibitor. While inflammatory cytokines are critical for memory CTLs formation, it is unknown if cytokines such as IL-12 mediate rapamycin's regulation during infection. Inhibition of mTOR by rapamycin represses CTL expansion but enhances central memory during vaccinia virus infection in mice. Without IL-12, immunoregulatory effects of rapamycin on CTL expansion and subsequent memory formation are diminished, yet present compared to CTLs not treated with rapamycin. In infected mice, rapamycin directly enhances IL-12 signaling in WT CTLs by upregulating IL-12 receptor-β2 and STAT4 phosphorylation. Furthermore, secondary expansion of rapamycin-regulated memory CTLs in IL-12 receptor knockouts

is impaired and resultant secondary memory CTLs are abolished. This indicates that interplay between cytokines and adjuvants should be considered during vaccine design.

THE ROLE OF INTERLEUKIN-12 FOR MTOR REGULATION OF MEMORY CTLS.

By

Karla Garcia

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Dedication

This thesis is dedicated to my family

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List of Abbreviations

Ab	Antibody
Ag	Antigen
APC	Antigen-presenting cell
B6	C57BL/6 mouse
B7	Peripheral membrane protein on activated APC cells
BM	Bone marrow
CCR7	Chemokine receptor 7
CD	Cluster of differentiation
CD62L	L-selectin
CD127	Interleukin-7 receptor-α subunit
CFU	Colony-forming unit
cTEC	Cortical thymic epithelial cells
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DN	Double negative cells
DP	Double positive cells
dsRNA	Double stranded RNA
EDTA	Ethylenediaminetetraacetic acid
EOMES	Eomesodermin
ERK	Extracellular signal-related kinase
FBS	Fetal bovine serum
H2-K^B	A classical MHC I haplotype present in B6 mice

HIV	Human immunodeficiency virus
IFN-γ	Interferon-gamma
IL	Interleukin
IL-12RKO	OT-I mouse deficient of IL-12 receptor β1
i.p.	Intraperitoneal
i.v.	Intravenous
JAK	Janus kinase
KLRG1	Killer cell lectin-like receptor subfamily G member 1
LCMV	lymphocytic choriomeningitis virus
LM-OVA	Listeria Monocytogenes expressing OVA
LN	Lymph node
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
mTOR	mammalian target of rapamycin
mTORC	mTOR complex
OTI	Transgenic mouse with CD8+ T cells that express a TCR specific for the SIINFEKL peptide of chicken ovalbumin
PBL	Peripheral blood
PBS	Phosphate buffered saline
PDK1	Phosphoinostide-dependent kinase 1
PFU	Plaque forming unit
PI	Post-infection
PI3K	Phosphoinositide 3-kinase

PIP	Phosphatidylinositol
PMBC	Peripheral blood mononuclear cell
Rapa	Rapamycin
RHEB	Ras homolog enriched in brain
s.c.	Subcutaneous
SOCS	Suppressor of cytokine signaling
SP	Single positive
STAT	Signal transducer and activator of transcription protein
T-bet	T-box transcription factor TBX21
T_{cm}	Central memory T cell
TCR	T cell receptor
T_{em}	Effector memory T cell
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
TNFR	Tumor necrosis factor receptor
TSC	Tuberous sclerosis complex
TYK	non-receptor tyrosine-protein kinase
VV-OVA	Recombinant Vaccinia virus expressing $OVA_{257-264}$
WT	Wild type

Chapter 1: Introduction

Significance

Despite the advances made in vaccination, infectious diseases are still one of the top causes of fatality, resulting in over 15 million deaths worldwide per year (1). Vaccination has virtually eliminated smallpox and polio, but infectious diseases such as malaria and HIV are still prevalent (2). Traditional vaccines cannot offer protection against some chronic infectious diseases like HIV, because these pathogens have an ability to hide within the host cell, or mutate, and replicate quickly (3). To tackle these obstacles, a new approach is necessary to produce effective vaccines against these problematic diseases. While functional memory cytotoxic T lymphocytes (CTLs) (also called cytotoxic $CD8^+$ T cells) are able to combat the invasion of intracellular pathogens, vaccine development to promote this process remains a major challenge (2, 4).

Vaccines are designed to ultimately generate protective immune memory, and specifically soliciting memory CTLs can result in a stronger response to recurring pathogens (2, 4). Activation of CTLs involves several simultaneous interactions between surface molecules on both CTLs and antigen-presenting cells (APCs) (2, 5). The formation of functional short-lived effector CTLs and long-lived memory CTLs requires stimulation from an antigen, costimulatory molecules, and a third signal provided by soluble cytokines such as interleukin-12 (IL-12) or type I interferon (5-13). The differentiation fate of effector and memory CTLs is regulated by a shift in balance of two cooperating transcription factors, T-bet and Eomesodermin (eomes)

(14). Additionally, mTOR (mammalian target of rapamycin), the master regulator of metabolism and cell growth, also plays a central role in CTL activation (15, 16). Interestingly, inhibition of mTOR by the immunosuppressive drug rapamycin can lead to enhanced memory CTL induction in *lymphocytic choriomeningitis* virus (LCMV) infections (17). It has been shown that the combination of rapamycin and IL-12 can interact *in vitro* to shift the balance from T-bet to Eomesodermin expression (14). However, whether important inflammatory cytokines such as IL-12 are involved in mTOR regulation of memory CTLs during infection is unknown. Therefore, the objective of my research is to determine whether mTOR's regulation of memory CTLs is dependent on IL-12 during a viral infection.

Immunity

Immunological protection from pathogens is provided by the innate and adaptive immune systems. Innate immunity evokes a rapid, non-specific response, while adaptive immunity provides specificity. Although both contribute to the elimination of foreign pathogens (2), a central feature of adaptive immunity is immunological memory. This produces a heightened response to recurrent invaders and is the ultimate goal of vaccination (2, 5). Immunological memory can be derived from two branches of immunity that cooperate during the adaptive immune response. Humoral immunity occurs when B cells interact with antigens and differentiate into antibody-secreting plasma cells that are capable of neutralizing extracellular pathogens. The cell-mediated immunity consists of T cells that recognize antigens through T-cell receptors (TCRs), and the antigens are presented by antigen presenting cells (APC) (2). T cells express one of two surface markers, CD4 or CD8. $CD4^+$ T

cells assist in the activation of B and $CD8^+$ T cell, while $CD8^+$ T cells, or CTLs, are responsible for direct destruction of pathogen-infected and tumor cells (2, 5).

CTL development begins when T-cell precursors migrate from the bone marrow and blood to the thymus to differentiate and develop into mature T cells (2, 4). Within different microenvironments of the thymus, T-cell precursors undergo a series of developmental changes defined by phenotypic alterations of the cell surface. Early T cells that lack CD4 and CD8 are double-negative (DN) cells, and are subdivided based on the presence or absence of other cell surface molecules during four different DN stages (DN1-4). Along the way, T-cell precursors commit to a T-cell lineage and rearrange their TCR gene loci and become double positive (DP) cells (CD4+ CD8+) after the final stage of DN4 (2). In the thymic cortex, DP cells come in contact with cortical thymic epithelial cells (cTECs) and are either negatively selected due to high major histocompatibility complex-to-peptide binding affinity, or positively selected to mature into single positive (SP) cells because of their intermediate binding affinity (2). Naïve CTLs then leave the thymus and enter into the circulatory system (4).

CTL activation

During an infection, the CTL must receive three signals that allow it to gain full functionality. APCs present pathogenic antigens in the form of a complex with their major histocompatibility complex class I (MHC-I) surface protein (2, 5). The antigen-specific binding of the CTL's TCR and co-receptor with the MHC-I bound antigen provides the first signal for CTL activation (18). Interaction between the costimulatory receptors (e.g. CD28 and some TNFR family members) on the CTL

and their corresponding costimulatory ligands (e.g. B7 family members) on APC provides the second signal (18). The first and second signals initiate a transduction cascade that allows for the activation of transcription factors and cytokines that coordinate CTL proliferation and survival (2, 5). Cytokines such as interleukin-12 (IL-12) and type I interferon alpha provide the third signal that increases clonal expansion and promotes full activation of naïve CTLs (5-10).

During primary response, CTLs acquire unique functionalities allowing them to either directly or indirectly remove pathogens. A large portion of CTLs differentiates into short-lived effector CTLs that directly combat infections by releasing killing-related molecules such as perforin and granzymes. Once the infection has subsided, a contraction phase ensues, during which 90-95% of the short-lived effector CTL die (9, 19, 20). The CTLs that survive the contraction phase become memory CTLs, which provide protection during a secondary response against recurring pathogens (9). These memory CTLs are formed in lymphoid tissues and perform their duties in secondary lymphoid organs or peripheral tissues (2). Memory CTLs provide long-lasting immunity to prevent recurring pathogens from mounting a potent infection. Upon subsequent challenge with the same antigen, memory CTLs display quick proliferation, and transition to effector cells to clear the pathogen effectively (21, 22). Effector (T_{EM}) and Central (T_{CM}) memory CTLs are two subpopulations of memory CTLs defined by their activated markers, chemokine receptors, and function (23, 24). T_{CM} express high levels of chemokine CCR7 and adhesion molecule CD62L, while T_{EM} are able to produce cytokines and express low levels of CCR7 and CD62L (9, 25). Memory CTLs are able to persist in part due to

signals delivered by two cytokines, IL-7 and IL-15 (9, 21, 26-28). Studies indicate that the function and phenotypic specificity of memory CTLs depend on both antigen and pro-inflammatory cytokines (21).

Interleukin-12

IL-12 is essential for programming memory CTLs in *vaccinia* virus infection as memory formation is substantially reduced when antigen-stimulated CTLs lack an IL-12 receptor (10). IL-12 is produced by macrophages, B cells, and dendritic cells during infections to induce interferon-gamma (IFN- γ) production (29). IL-12 is a heterodimer composed of two subunits, IL-12p35 and IL-12p40. On the CTL surface, IL-12p35 and IL-12p40 bind to IL-12Rβ2 and IL-12Rβ1, respectively. As a result, JAK2 and TYK2 are phosphorylated via tyrosine phosphorylation (29). The IL-12Rβ2 is also phosphorylated to function as a docking site for the signal transducer and activator of transcription 4 (STAT4). STAT4 becomes phosphorylated once it binds to the IL-12Rβ2 receptor subunit, forming STAT4 homodimers, which migrate to the nucleus and bind to the promoters of IFN-γ and other IL-12 responsive genes (2, 29). This signaling then induces transcription of IFN-γ, through mechanisms that are still not clearly understood (29). IL-12 is considered a natural adjuvant as it has the potential of enhancing the efficacy of vaccines. It has been shown to have dramatic effects on suppressing tumor growth in cancer therapies (30).

mTOR

In addition to requiring three signals for activation, CTLs also need nutrients from the body to function, making cellular regulation of nutrient uptake crucial. Recently, mTOR, a master regulator of metabolism and cell growth, was found to be directly involved in CTL activation (31). mTOR is an evolutionarily conserved 289-kDa serine-threonine protein kinase, which is part of the phosphoinositide 3-kinase family (32). Its ability to sense and integrate signals from the immune microenvironment makes mTOR a vital regulator of immune function. mTOR signaling occurs through two complexes, mTOR Complex 1 (mTORC1) and Complex 2 (mTORC2). mTORC1 is involved in the regulation of cellular transcription, translation, autophagy, and production of new ribosomes. On the other hand, mTORC2 possibly regulates cell survival and actin cytoskeletal structuring (32). In the immune system, mTOR activation begins with the recruitment of PI3 kinase (PI3K) by growth factors or cytokines, but mTORC1 can also be activated by other immunological factors or extracellular cues (33-35). PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2) to form phosphatidylinositol 3,4,5-triphosphate (PIP3), which directs Akt to the membrane where it is phosphorylated by phosphoinostide-dependent kinase 1 (PDK1). Activated Akt or extracellular signal-related kinases 1 and 2 (ERK1/2) phosphorylates tuberous sclerosis 2 (TSC2), separating it from the tuberous sclerosis complex (TSC1/TSC2). This leads to accumulation of GTP-binding Ras homolog enriched in brain (RHEB-GTP). The abundance of RHEB-GTP enhances mTORC1 function (33, 35, 36). Immunologically, mTORC1 activation leads to the downstream enhancement of

immune cells because of an increase in STAT3 and STAT4 through inhibition of SOCS3 (33, 35-37). Unlike its counterpart, mTORC2 is poorly understood and its upstream and downstream regulatory mechanisms are unclear (36).

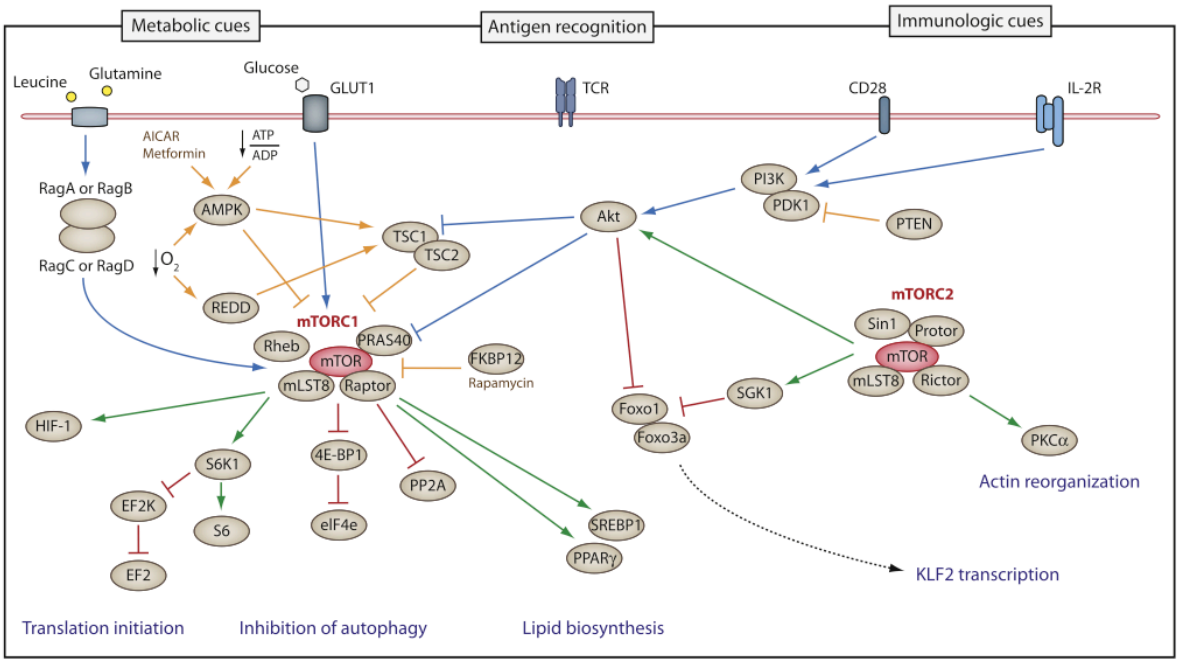


Figure 1[i]. mTOR Signaling for the Immunologist (35).

[i] Reprinted from *Immunity*, Vol 33/ Issue 3, Jonathan D. Powell and Greg M. Delgoffe, The Mammalian Target of Rapamycin: Linking T Cell Differentiation, Function, and Metabolism, Pages 301-311, Copyright (2010), with permission from Elsevier

Although IL-12 is known to provide the third signal for CTLs, its involvement in rapamycin's regulatory effects in animals is unknown. For this reason, we want to investigate whether IL-12 plays a role in the regulation of rapamycin on memory development during viral infection. Based on prior work conducted by the lab and other research groups (14, 17, 38), I hypothesize that in the presence of IL-12 signaling, rapamycin will enhance memory CTL response during *vaccinia* virus infection.

Chapter 2: Materials and Methods

Mouse Model

To conduct this research, we used transgenic mice containing OT-I cells with and without IL-12 receptor β1 deficiency (IL-12RKO). Mice containing OT-I cells have contain unique CTLs that posse TCRs specific for the SIINFEKL (amino acids 257 to 264 of the OVA antigen epitope) (10). C57BL/6 male mice were purchased from the National Cancer Institute. The mice were kept under specific pathogen-free conditions at the University of Maryland, which were approved by the Institutional Animal Care and Use Committee.

Infectious Agents

The recognition of the OVA antigen expressed on recombinant *vaccinia* virus (VV-OVA) and recombinant *listeria monocytogenes* (LM-OVA) causes the activation of the unique CTLs in mice containing OT-I cells (38).

Naïve T Cell Purification

Naïve OT-I cells were extracted from the inguinal, axillary, brachial, cervical, and mesenteric lymph nodes from both WT and IL12RKO mice. The cells were incubated together with FITC-labeled antibodies that are specific for B220, CD4, CD44, and I-A^b. The suspension was subsequently incubated with FITC binding magnetic MicroBeads (Miltenyi Biotech, Auburn CA), and passed through separation columns attached to a MACS magnet. Cells that did not bind to the column were collected with a purity >95% $CD8^+$ and <0.5% $CD44^{hi}$ cells.

Adoptive Transfer and Flow Cytomery Analysis

Then, purified OT-I cells were injected via the tail vein into C57BL/6 mice at 10^5 cells/mouse. The transferred OT-I cells were then identified as $CD8^+CD45.2^+$ cells. Viable cell counts were performed using trypan blue, and flow cytomerty was be used to determine the percentage of OT-I cells in the samples. After adoptive transfer, the recipient mice were infected with 5 x 10^6 PFUs of VV-OVA to solicit an immune response. Blood was drawn at different time points post-infection for memory CTL analysis with conjugated fluorescent antibodies.

Intracellular Staining

To perform intracellular cytokine staining, single cell suspension from adoptively transferred mice were incubated at 37^{o}C for 3.5 hours with 2 x 10^6 cells/ml in RP-10 with 0.2 uM $OVA_{257-264}$ peptide and 1 ul/mL Brefeldin A (Biolegend). Fixing buffer from Biolegened was added to the cells for 15 minutes at 4^{o}C, after which the cells were permeabilized in Saponin-containing Perm/Wash buffer (Biolegend) for another 15 minutes at 4^{o}C, then stained with PE-conjugated antibody to IFN gamma or APC-conjugated antibody to TNF alpha for 30 minutes at 4^{o}C. Finally, the cells were washed once with Perm/Wash buffer and PBS containing 2% FBS. The memory CTL population forms near the end of the contraction phase and remains stable for a few weeks after an acute viral infection or immunization (2). Therefore, a one-month cutoff was implemented to observe memory CTLs formation after the VV-OVA infection.

Administration of Rapamycin and Bacterial Infection

Rapamycin was administered to mice daily through i.p. injections during a defined treatment period. Two different treatment periods were used: 1) high dose (600 μg/kg) administered during VV-OVA infection (day -1 prior to infection to day 10 post-infection); 2) low dose (75 μg/kg) administered during VV-OVA infection (day -1 prior to infection to day 30 post-infection) (17). Control mice received no treatment.

After a month, the mice were challenged with an i.v. injection of LM-OVA at either 10^4 CFU/mouse (for secondary expansion of memory CTLs) or at 5 x 10^5 CFU/mouse (for memory CTL protection). Three days after being challenged with LM-OVA, mouse spleens were harvested for bacterial culture using TSB plates to compare protection abilities (10).

Tissue Harvest and Digestion

For tissue harvesting and digestions the mice were euthanized. Spleens and peripheral lymph nodes were removed and homogenized in 15 ml glass grinders. Lungs were first perfused with 1 x PBS at about 30 ml per mouse, cut into 1mm^3 size pieces, homogenized with a 10 ml pipette, and resuspended in 4 ml Collagenase D. To ensure complete digestion, lung tissues were kept in a 37^{o}C water bath for 25 minutes. Then to halt digestion, 0.1 M EDTA was added and those tissues were homogenized using glass grinders.

Statistical Analysis

Data was statistically analyzed using a two-tailed student's t test or with two-way ANOVA on GraphPad Prism 5.0 software. Comparisons that result in a P value <0.05 were considered significantly different.

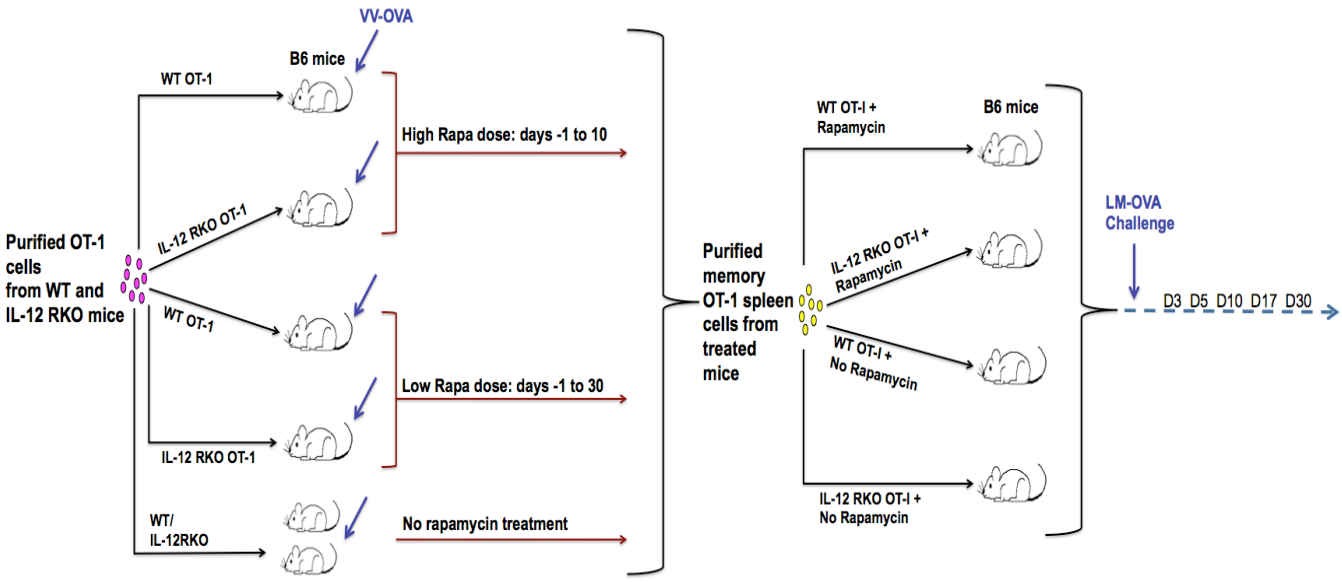


Figure 2. Scheme of experimental plan. Purified OT-I cells from WT and IL-12RKO mice were transferred into recipient B6 mice. The recipient B6 mice received one of three treatments starting a day before VV-OVA infection: 1) high dose (600 µg/kg) rapamycin administered from day -1 prior to infection to day 10 post-infection; 2) low dose (75 µg/kg) rapamycin administered from day -1 prior to infection to day 30 post-infection. Control B6 mice received no treatment. Samples were collected at days 5, 10, 17, and 30 post VV-OVA infection. At day 30, treated B6 mice were euthanized and memory OT-I cells were purified from spleens and transferred into a group of B6 mice. Spleen and blood samples were collected at days 3, 5, 10, 17, and 30 after LM-OVA infection.

Chapter 3: Results[ii]

Rapamycin enhances memory CTLs during VV infection

Administration of rapamycin to mice can promote memory CTLs in both LCMV (17) and LM infections (39). 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) (40). 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 (38). In addition, high doses of rapamycin can accelerate the transition of effectors to memory CTLs in LCMV infection (17). We speculated that daily administration of high doses of rapamycin early in infection would be immunostimulatory, as this period corresponds to memory CTL programming by IL-12 *in vitro* (38). 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 (Figure 4). Memory OT-I cells were examined at D30 postinfection (PI). Consistent with the report by Araki et al. (17), inhibition of mTOR by rapamycin significantly enhanced memory CTLs during VV infection by fourfold when administered from D−1 to D10 PI (Figure 3a and Figure 5). 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 3a). Thus, we used D−1 to D10 PI as

[ii] Reprinted by permission from Macmillan Publishers Ltd: *Genes and Immunity,* advance online publication, 5 June 2014; doi: 10.1038/gene.2014.33

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 (17). 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 (17). Similar to LCMV infection (17), rapamycin upregulated CD62L expression in memory CTLs (Figure 3b). In addition, bulk splenocytes containing an equal number of memory OT-Is (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 (41, 42). Memory OT-I cells generated with and without rapamycin achieved similar protection (Figure 3c). 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^b/OVA tetramer was used to detect endogenous OVA-specific CD8 T cells (40). We confirmed that rapamycin promoted endogenous memory CTLs similar to memory OT-I cells (Figure 3d). CD62L was upregulated in the rapamycin-treated

endogenous memory K^b/OVA-positive CTLs (Figure 3e). 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.

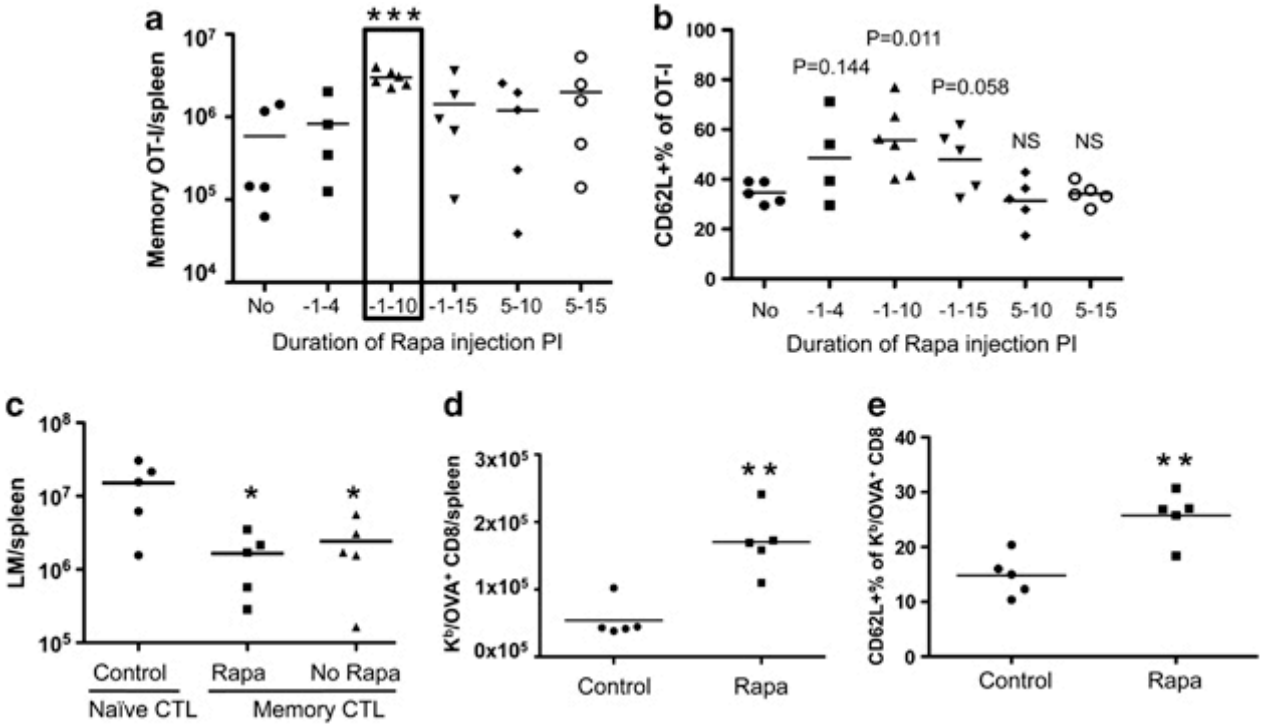


Figure 3. Rapamycin enhances memory CTLs during VV infection. Purified naive OT-I cells were transferred into naive B6 recipients, which were infected with VV-OVA the next day. Rapamycin was injected daily at 600 μg kg^{-1} through intraperitoneal at the time windows indicated in panel (a). (a) Memory 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^bOVA^+ 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^bOVA^+ 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.

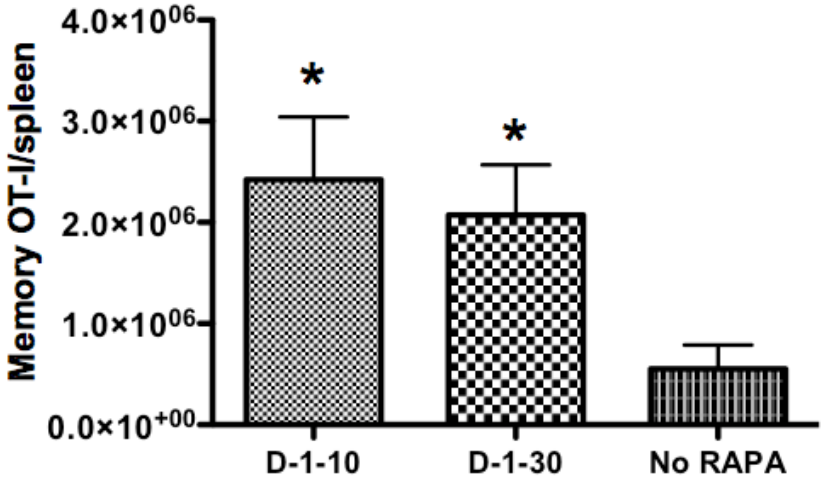


Figure 4. Rapamycin enhances memory CTLs during vaccinia virus infection. Purified naïve OT-I cells were transferred into naïve B6 recipients, which were infected with VV-OVA the next day. Rapamycin was injected daily at 600ug/kg through i.p at the time windows as indicated. Memory OT-I cells from spleens were examined on 40 days post-infection (PI). Student's *t* test was performed comparing each of the groups with no rapamycin controls (No RAPA) *, $P < 0.05$. The bars represent the mean of six animals plus SEM.

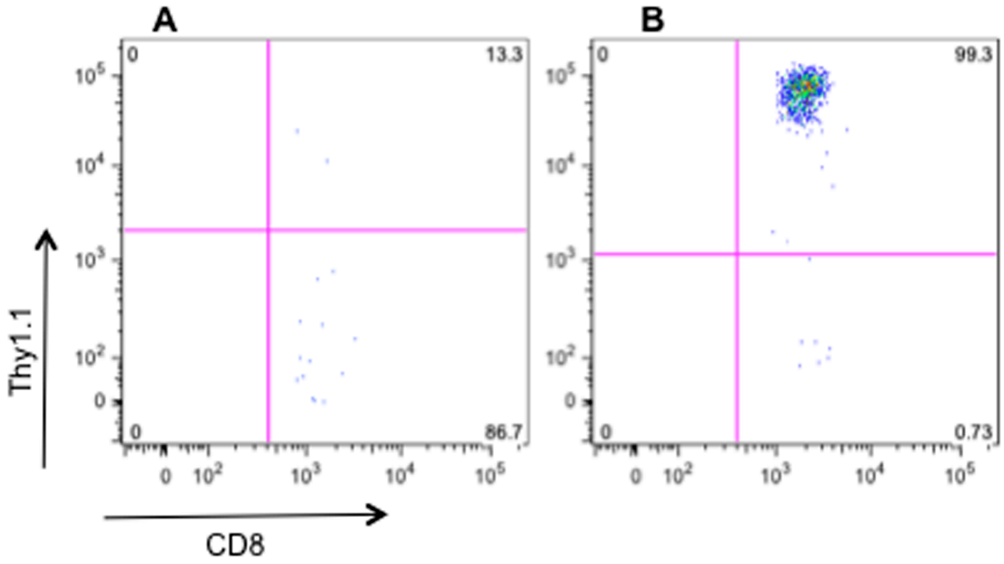


Figure 5. Detection of endogenous K^b/OVA CD8 in OT-I transferred memory mice. Purified naïve OT-I cells were transferred into naïve B6 recipients, which were infected with VV-OVA the next day. Rapamycin injection was from day -1 to 10 post-infection. CTLs were detected in the spleen from naïve (a) or memory mice (b) (30 days post-infection). The dot plots were cells gated on K^b/OVA tetramer-positive and CD8-positive. Data are representative of three experiments with similar results.

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 (41) (Figure 7) 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 3. 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 6). This is consistent with the report that a high dose of rapamycin inhibits expansion of effectors in LCMV infection (17). However, CTLs significantly expanded between D5 and D10 in the rapamycin-treated WT and IL-12RKO groups (Figure 6a), and this expansion accelerated upon withdrawal of rapamycin until day 17. Notably, WT OT-Is expanded almost two times more than IL-12RKO OT-Is (Figure 6b) 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 (38). After D17, the CTL population contracted, and a fraction of expanded cells became memory CTLs at D30, remaining stable thereafter (Figure 6a and data not shown). WT OT-Is contracted more than IL-12RKO OT-Is, based on lower expansion of IL-12RKO (Figure 6c). 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 6d), which is consistent with its effects during *in vitro* stimulation (38). The

continued expansion of OT-Is upon the withdrawal of rapamycin led to a quick downregulation of CD62L, although expression of CD62L remained higher than in their untreated counterparts (Figure 6d). 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-Is 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 6d). Furthermore, IL-7 receptor α (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 6e). 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 6f). These data suggest that rapamycin favors a central memory CTL phenotype (CD62Lhi/CD127hi/KLRG1lo), and the IL-12 signal may contribute to this phenotype.

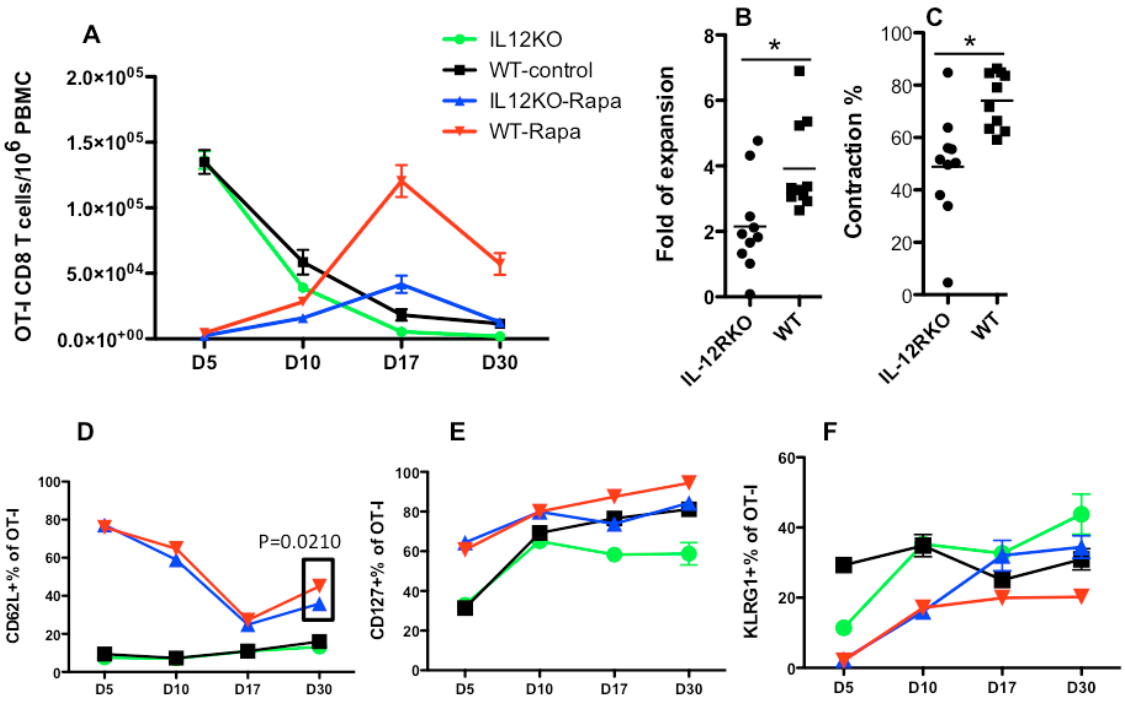


Figure 6. IL-12 increases CTL expansion after rapamycin treatment. OT-I cells were purified from WT or IL-12RKO OT-I mice, which were transferred into naive B6 mice at 10^5/mouse through tail vein. Recipients were infected with VV-OVA the next day. Daily rapamycin injection occurred from D−1 to D10 PI. (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-Is 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.

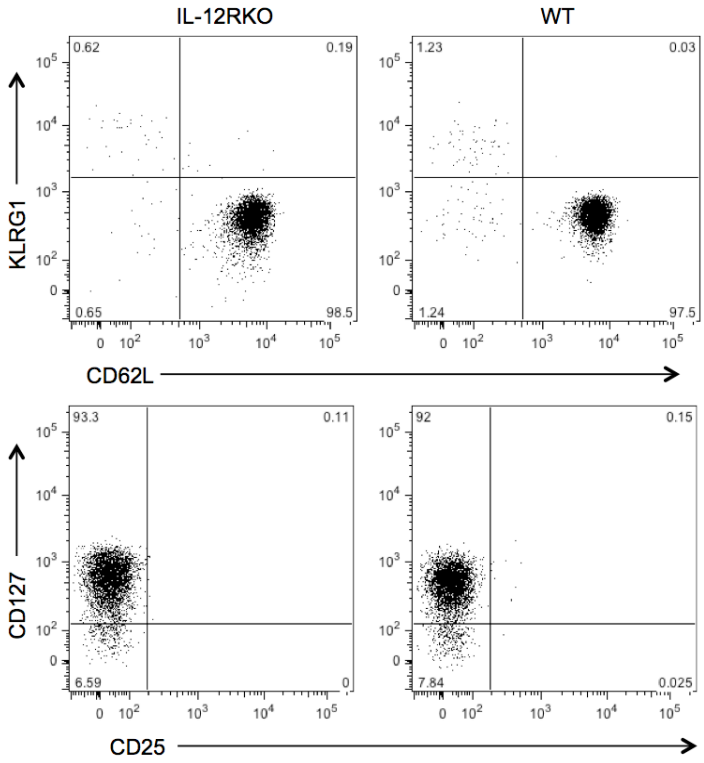


Figure 7. Comparison of CD8 T cells from WT and IL-12RKO OT-I mice. CD8 T cells in blood were examined for expression of several surface molecules. WT: WT OT-I mice. IL-12RKO: IL-12RKO OT-I mice.

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-Is in tissues compared with corresponding controls (Figure 8a). 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 8a).

To investigate whether rapamycin altered migration of memory CTLs, we analyzed the tissue distribution of memory OT-Is. Although rapamycin treatment increased the number of memory OT-Is in tissues in both WT and IL-12RKO (Figure 8b), rapamycin-regulated memory OT-Is tended to remain in the spleen (P=0.057) compared with CTLs not treated with rapamycin (Figure 9a). This trend disappeared in IL-12RKO OT-Is (P=0.578), which were retained in the spleen at similar percentages regardless of the exposure to rapamycin (Figure 9a 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 (Figure 9a), consistent with the observation of enhanced central memory phenotype due to rapamycin. The memory OT-Is in the spleens from rapamycin-treated mice exhibited increased expression of CD62L when compared with WT controls (Figure 8c and d). Similar to blood samples (Figure 8e 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 (Figure 8e and f). These observations were similarly reflected in memory OT-Is from most tissues (some differences were not significant), although the expression levels varied among tissues in the same animals (Figure 9c-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 (Figure 9c-e). These results suggest a general trend: rapamycin promotes a central memory phenotype of CTLs in tissues and in the periphery.

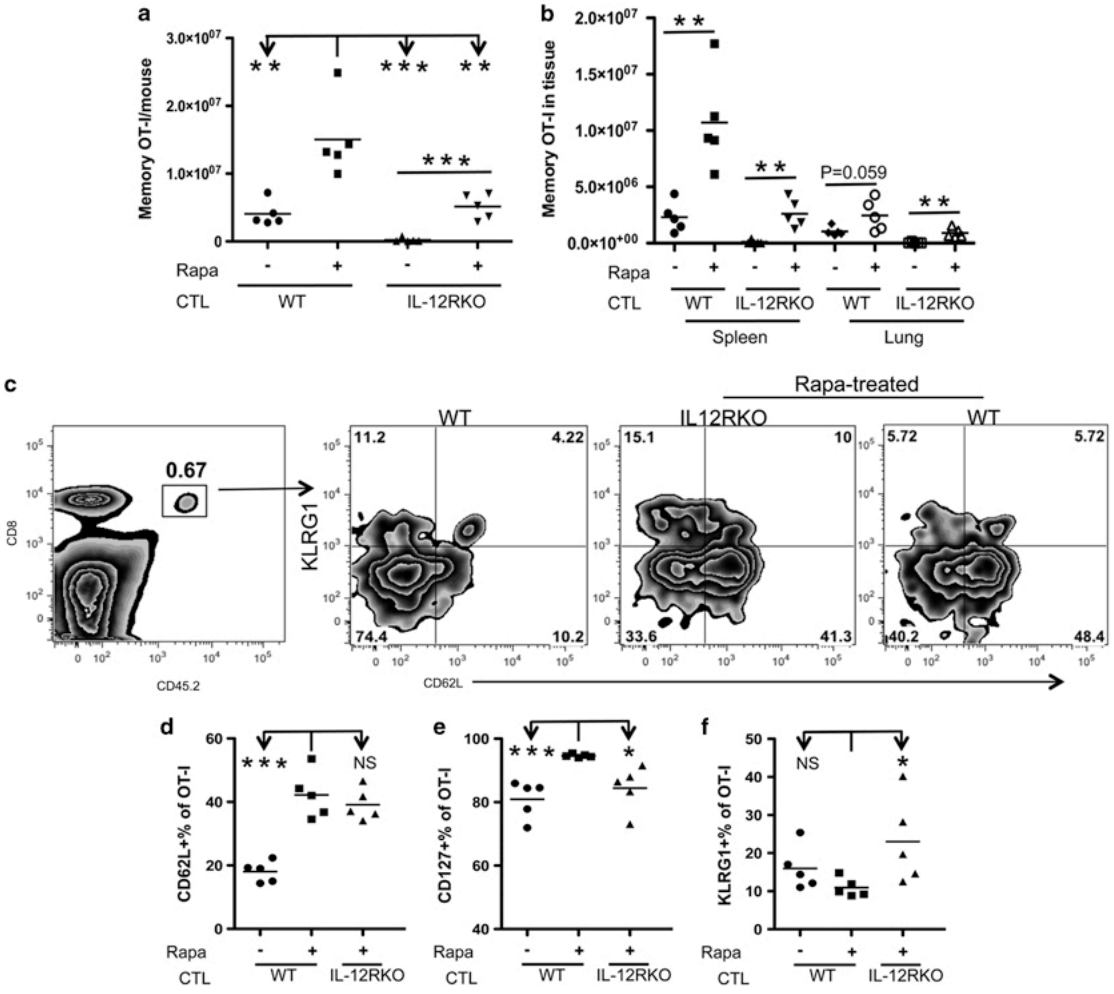


Figure 8. Rapamycin enhances memory CTLs in tissues. Memory OT-I cells were analyzed in memory mice (similar to those in Figure 6a) 40 days after VV-OVA infection. (a) Comparison of total memory OT-I cells from the peripheral LNs, spleen, lung and two sets of femur from each mouse. (b) Tissue distribution 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.

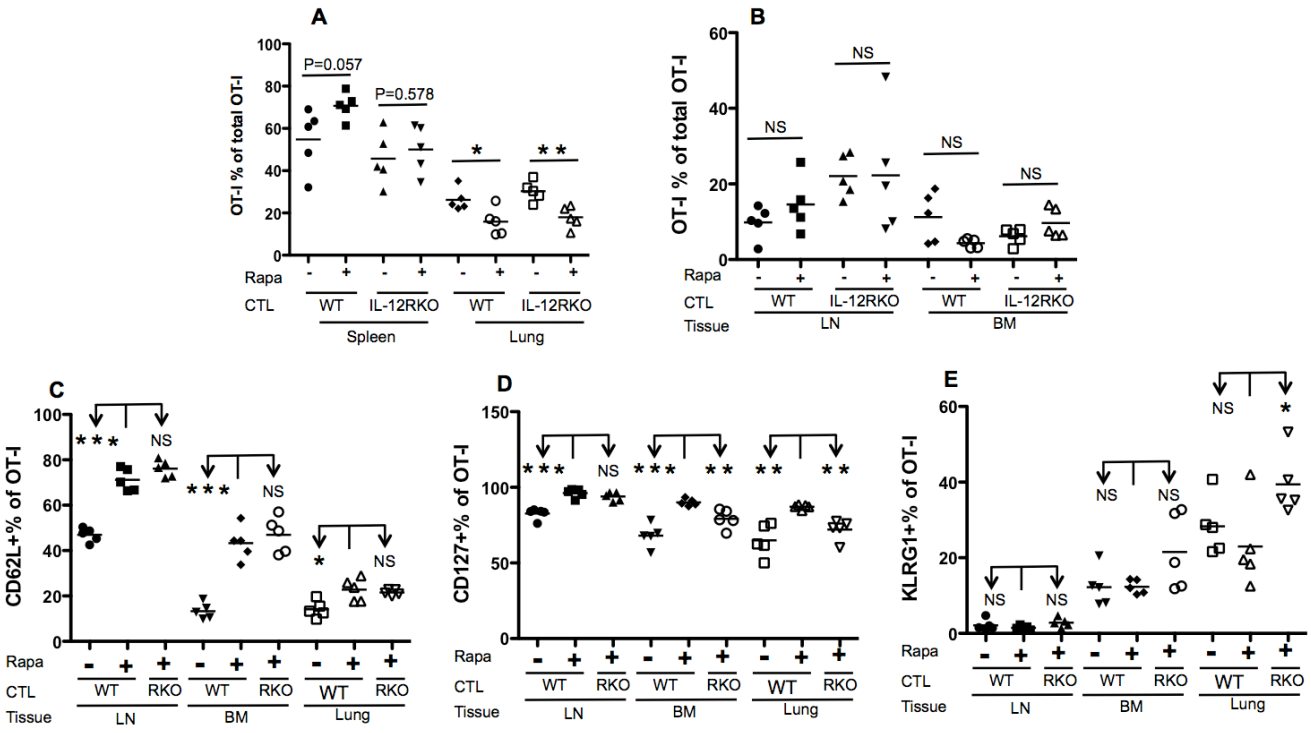


Figure 9. Memory CTLs are enhances in tissues by rapamycin. Memory OT-I cells were analyzed in memory mice (the same mice in Figure 3a) 40 days after VV-OVA infection. A-B. Tissue distribution of memory OT-I cells. Data were calculated by dividing the number of memory OT-I in one tissue by the number in all examined tissues. C-E. Representative expression of CD62L/CD127/KLRG1, and corresponding statistics (Student's *t* test), in memory OT-I cells in tissues from mice in (A). The experiment was repeated three times and similar results were obtained.

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 chronic LCMV infection (43-45). To test whether the CTLs in this study were functional, memory mice were challenged with LM-OVA (38, 41). The memory mice that had originally received IL-12RKO OT-Is were not protected against LM-OVA challenge, as is consistent with our previous report (41) (Figure 10a). 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 10a). Endogenous K^b/OVA CD8 T cells were undetectable (data not shown), suggesting that memory IL-12RKO OT-Is were responsible for the enhanced memory protection in IL-12RKO OT-I transfer mice. IFN-γ and tumor necrosis factor α (TNFα) 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 (Figure 10b-d). Notably, there were significant differences in IFN-γ and TNFα production by memory CTLs from different tissues within the same individual: CTLs in the lungs produced the lowest amount of IFN-γ and TNFα, whereas CTLs in the spleens, LNs and bone marrow produced more of these cytokines (Figure 10c 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.

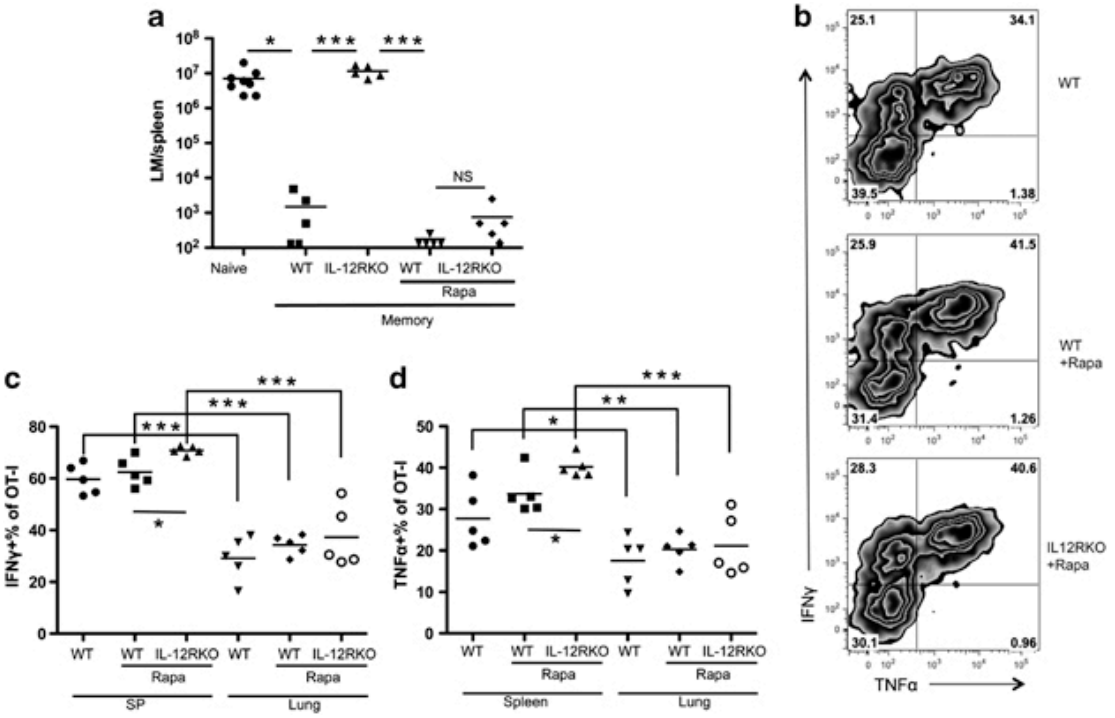


Figure 10. Rapamycin-regulated memory CTLs are functional in the absence of the IL-12 signal. (a) Memory mice (similar to those in Figure 6a) were challenged with LM-OVA, and bacterium was recovered from the spleen 3 days after challenge. (b–d) Resting memory OT-I cells in different tissues were examined for the production of IFN-γ and TNFα. 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.

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 (1, 21). To test secondary expansion ability, an equal number (10^5) of memory OT-Is from each treatment group was transferred into naive recipients, which were then challenged with LM-OVA. OT-Is became detectable at D5, peaked at D7 and contracted thereafter (Figure 11a). IL-12RKO OT-Is had the smallest expansion at D7, which was significantly lower than the other groups (Figure 11b). Furthermore, this group (IL-12RKO) contracted the most, becoming almost undetectable at D14 postchallenge (Figure 11a). Interestingly, rapamycin-regulated WT memory OT-Is were significantly lower than WT memory controls at D7 (Figure 11b), but both achieved a similar level of secondary memory (D30 after re-challenge Figure 11a). 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 11c and Figure 12a-c) and D5 (data not shown). The extent of expansion was predictive of the resultant secondary memory: secondary memory CTLs were undetectable in the IL-12RKO+ rapamycin group (Figure 11d). Secondary memory from either WT memory or WT+ rapamycin memory CTLs was higher than in naive controls (Figure 11d). 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 11e). Collectively, lack of the IL-12 signal causes defective secondary expansion and abolishes secondary memory formation.

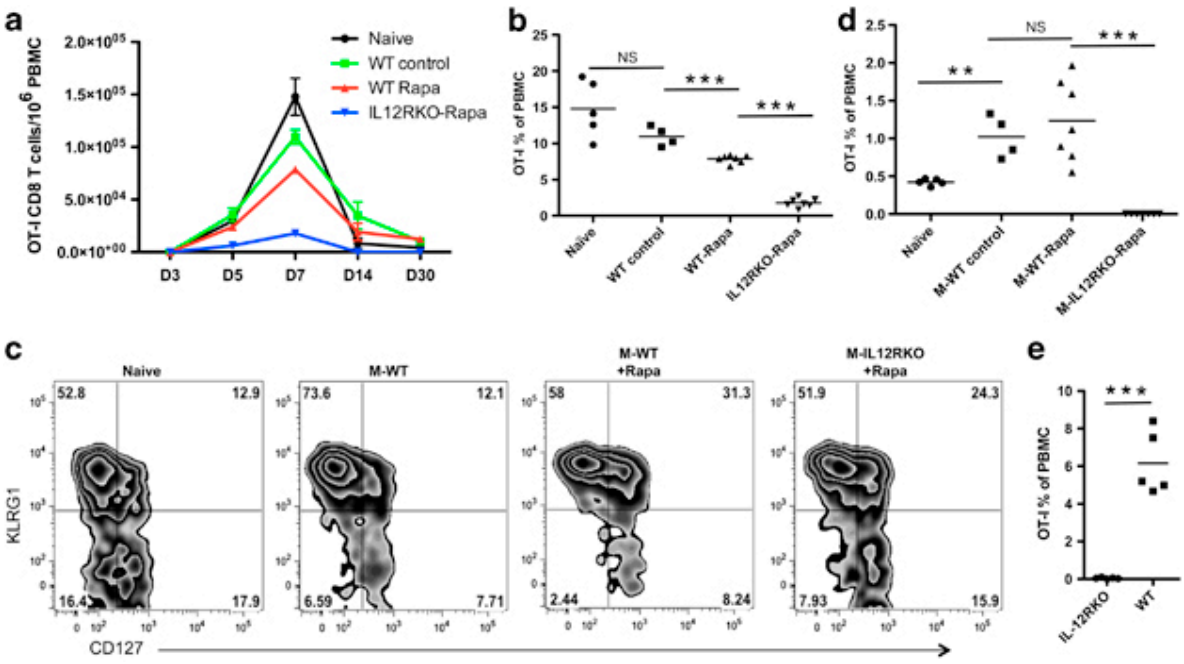


Figure 11. IL-12 is required for secondary expansion of memory CTLs regulated by rapamycin. Naive mice having received naive or IL-12RKO OT-I cells were split into two groups: rapamycin-treated and untreated control. These mice were then infected with VV-OVA. Splenocytes containing 10^5 memory OT-I cells from each of the treatments were transferred into naive B6 mice, which were challenged the next day with LM-OVA. Memory IL-12RKO OT-Is 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-Is at D7 after LM-OVA challenge. (e) Mice that have received rapamycin-treated first memory OT-Is (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).

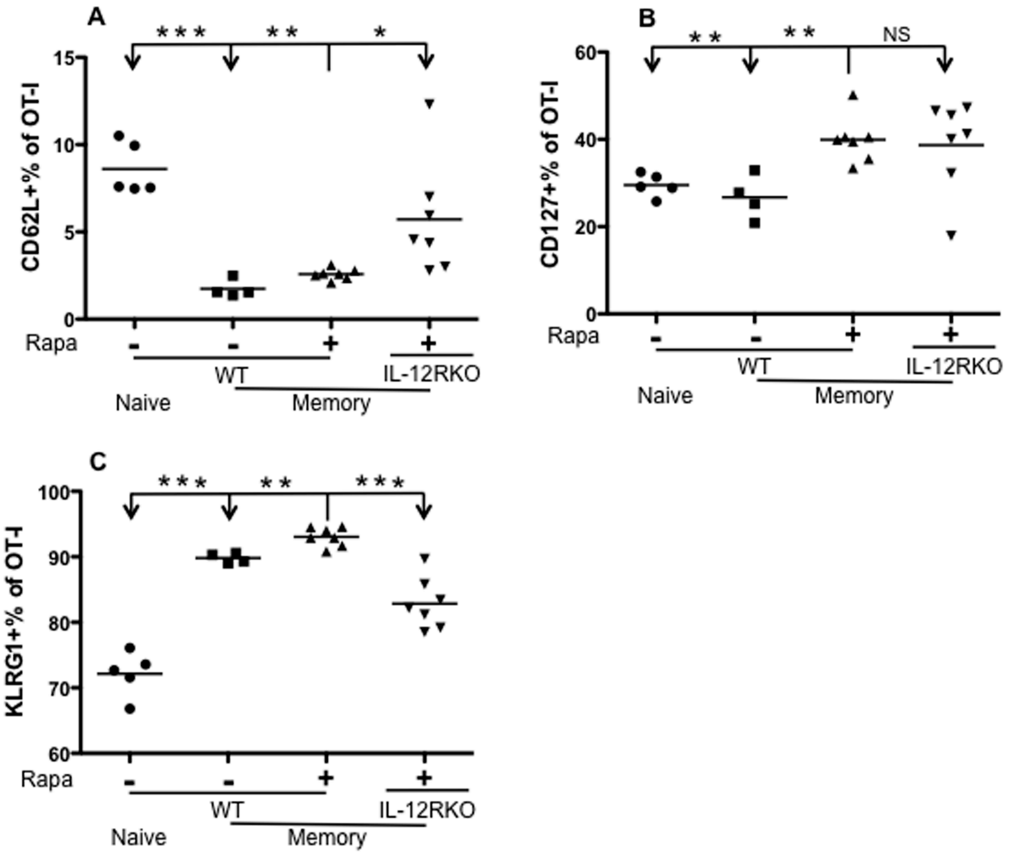


Figure 12. Secondary expansion of memory CTLs regulated by rapamycin requires IL-12. Naïve mice having received naïve or IL-12RKO OT-I cells were split into two groups: rapamycin-treated and untreated control. These mice were then infected with VV-OVA. Splenocytes containing 10^5 memory OT-I cells from each of the treatments were transferred into naïve B6 mice, which were challenged the next day with LM-OVA. Memory IL-12RKO OT-Is without rapamycin were at or below detectable level, so were excluded in transfer. OT-I populations were tracked in blood samples. A-C. Comparison of expression of KLRG1/CD127/CD62L in OT-Is at day 7 after LM-OVA challenge. The results are representative of two separate experiments with similar results. Student's *t* test was performed.

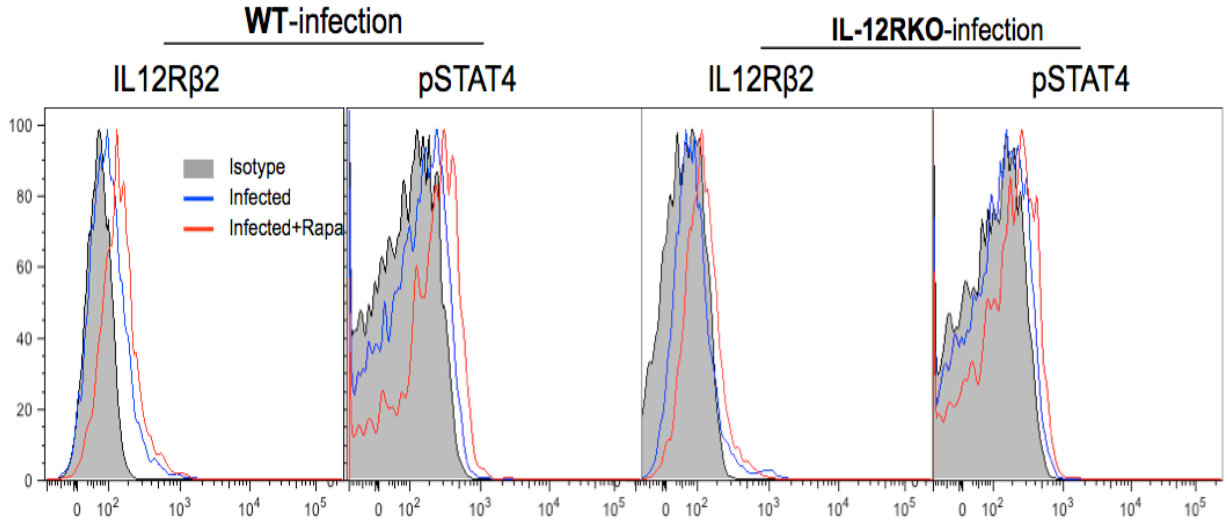
Rapamycin enhances IL-12 signaling in early infection and consistently inhibits T-bet expression

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 6. OT-Is were analyzed for IL-12 signaling and other pathways at different time points PI. IL-12 receptors are composed of two subunits, β1 (shared with IL-23) and β2 (binding p35 of IL-12, so is unique to IL-12) (46-48). Our IL-12RKO OT-Is are deficient in the β1 subunit. β1 and β2 are differentially expressed in immune cells (2). In naive CD4 cells, β1 is expressed but β2 is absent (49). The expression of β2 is induced by IFN-γ but inhibited by IL-4 during activation (49). In CD8 T cells, both β1 and β2 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 β2 was upregulated earlier and with greater magnitude than was β1 (50). Administration of rapamycin increased IL-12R β2 expression in both WT and IL-12RKO OT-Is during early infection (days 3–5) but not β1 (Figure 13a and data not shown). Type I IFN receptor subunit 1 was not affected by rapamycin (Figure 14a). This indicates that IL-12R β2 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 13a). This suggests that rapamycin directly enhances STAT4 activation

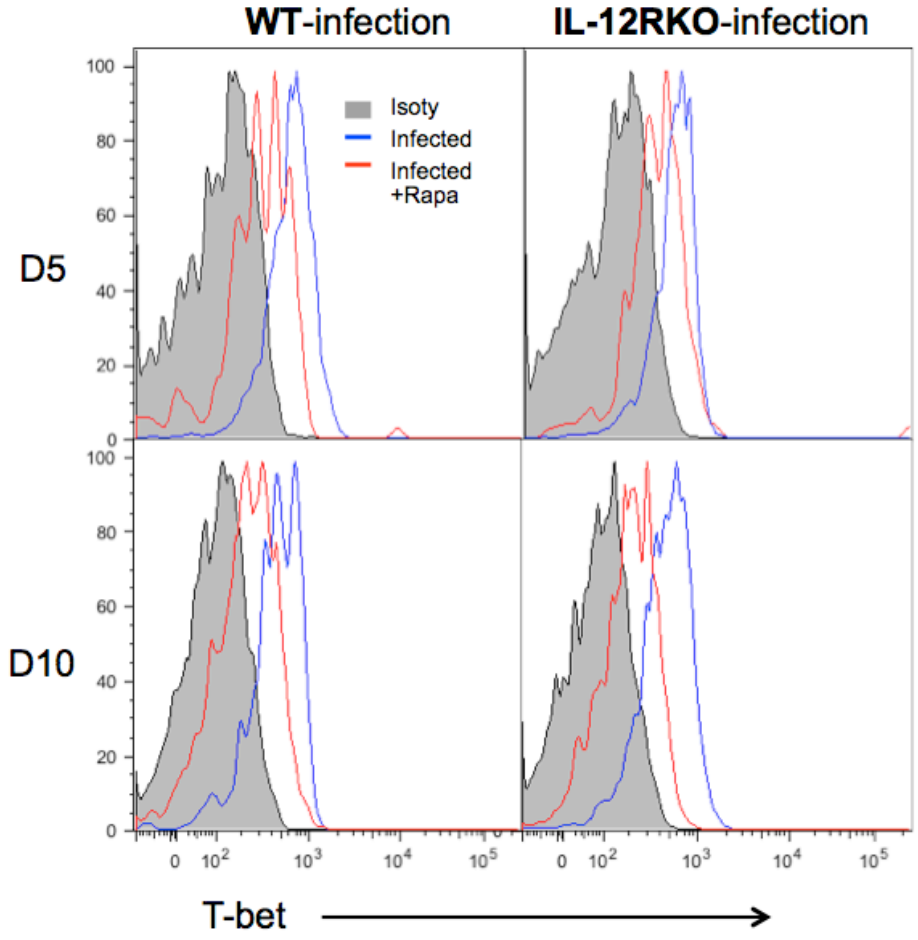
during early infection through the IL-12 signaling pathway and/or other cytokines (41, 51-53). T-bet is a transcription factor responsible for CTL effector function (54, 55). Rapamycin regulates IL-12-driven memory programming by inhibiting T-bet and promoting Eomes expression (14). Consistent with this, administration of rapamycin suppressed T-bet expression in both WT and IL-12RKO CTLs at days 5 and 10 PI (Figure 13b and data not shown), but Eomes expression was not affected (Figure 14b). Thus, rapamycin's suppression of CTL effector function may contribute to the enhanced memory in both WT and IL-12RKO OT-Is. Interestingly, mTOR phosphorylation was not altered by rapamycin at days 5 and 10 PI, indicating that rapamycin may work through pathways other than mTOR (Figure 14c). Therefore, our findings suggest that rapamycin can both directly augment IL-12 signaling during early infection and suppress CTL effector function.

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 (38, 56). Indeed, rapamycin directly enhanced and extended STAT4 phosphorylation when IL-12 was present (Figure 13c). Consistent with the data in VV infection (Figure 13a), rapamycin directly inhibited T-bet expression independent of IL-12 (Figure 13c) as previously reported (14). In contrast to *in vivo*, IL-12Rβ2 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.

A


WT-infection
IL-12RKO-infection
IL12Rβ2
pSTAT4
IL12Rβ2
pSTAT4
Isotype
Infected
Infected+Rapa

B


WT-infection
IL-12RKO-infection
Isoty
Infected
Infected
+Rapa
D5
D10
T-bet

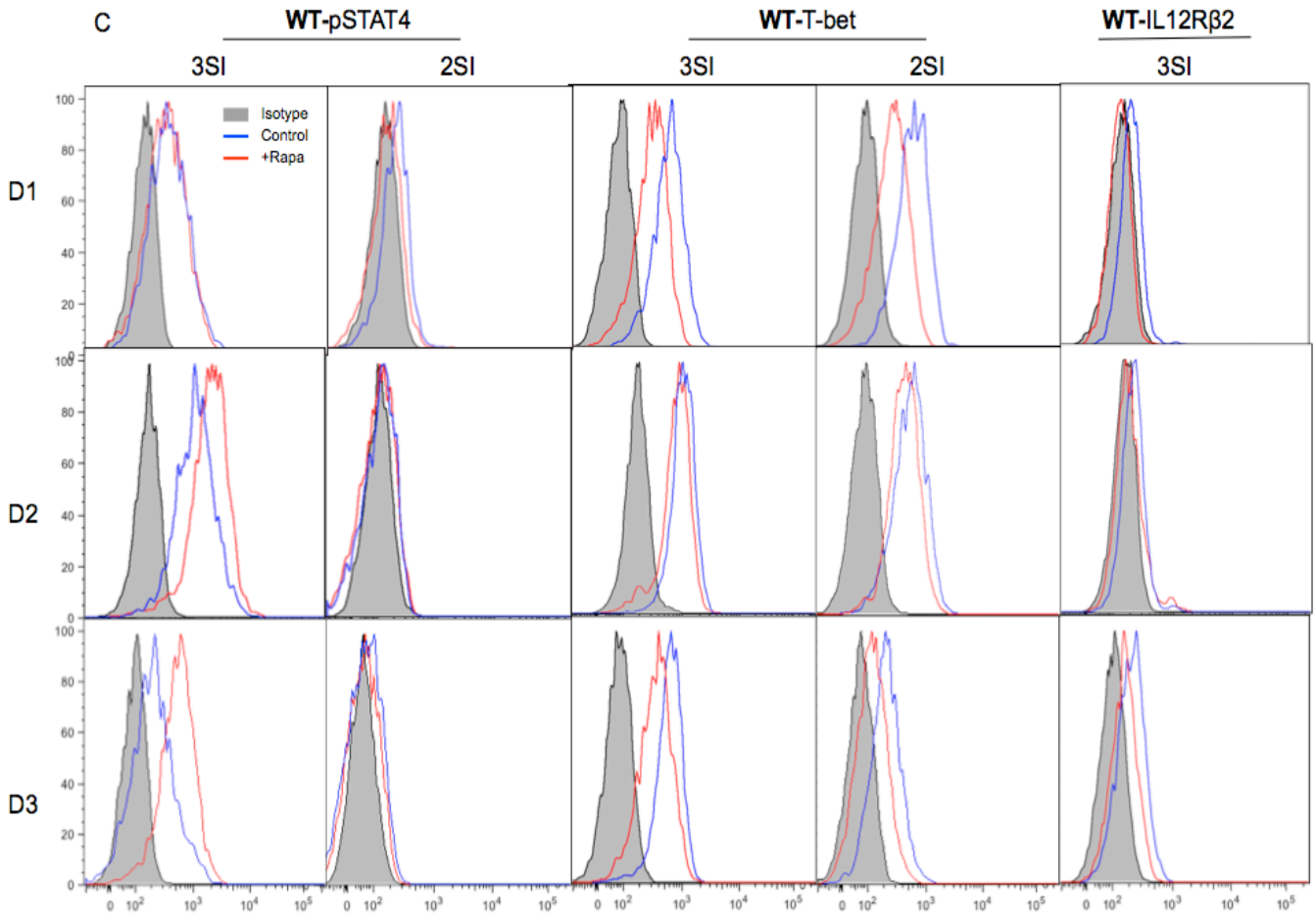


Figure 13. Rapamycin enhances IL-12 signaling in early infection and consistently inhibits T-bet expression. Naive WT or IL-12RKO OT-I cells were transferred into recipient B6 mice, which were infected with VV-OVA the next day. High doses of rapamycin were administered daily between D−1 and D10 after 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 (38). 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.

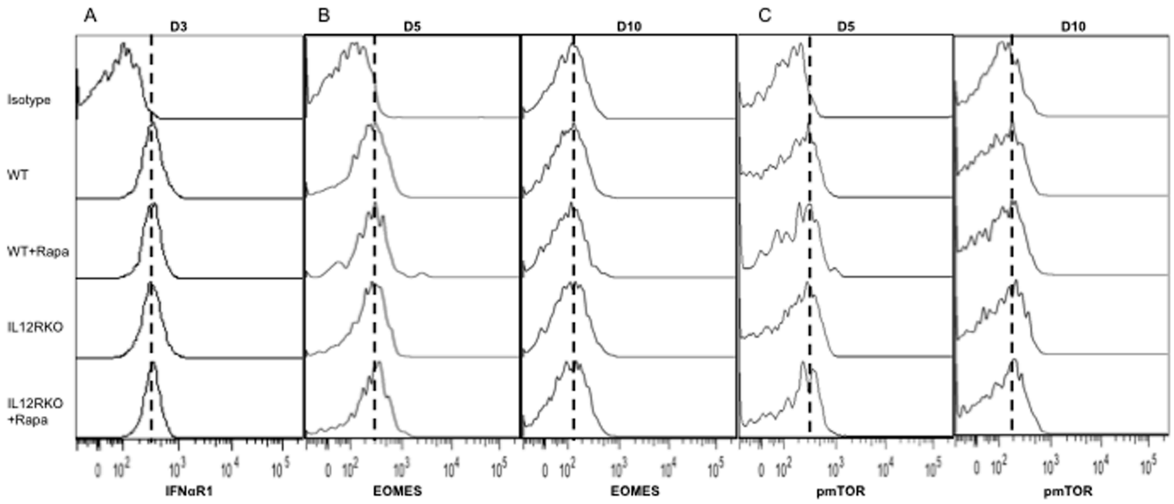


Figure 14. Effetcs of rapamycin on IFNαR1, EOMES and mTOR. Naïve WT or IL-12RKO OT-I cells were transferred into recipient B6 mice, which were infected with VV-OVA the next day. High doses of rapamycin were administered daily between days -1 and 10 post VV-OVA infection. OT-I cells in spleens were examined at days 3, 5 and 10 after infection. A. Comparison of IFNαR1 expression at day 3 post infection. B. Comparison of EOMES expression at days 5 and 10 post infection. C. Comparison of mTOR activation at days 5 and 10 post infection. The results are representative of 5 mice per group, and similar data were obtained in two separate experiments.

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 (17). 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-12RKOs (Figure 15a). Yet, low doses of rapamycin inhibited CTL expansion in both WT and IL-12RKO CTLs, albeit more in IL-12RKOs (Figure 15a). 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 15b), 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 15b). Despite the differences in expansion and expression of surface molecules, memory CTLs reached similar levels in both WT and IL-12RKO OT-Is regardless of the dose of rapamycin (Figure 15c). Furthermore, CTLs from both doses tended towards central memory phenotype—CD62L positive and mostly KLRG1 negative and CD127 positive (Figure 15d 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.

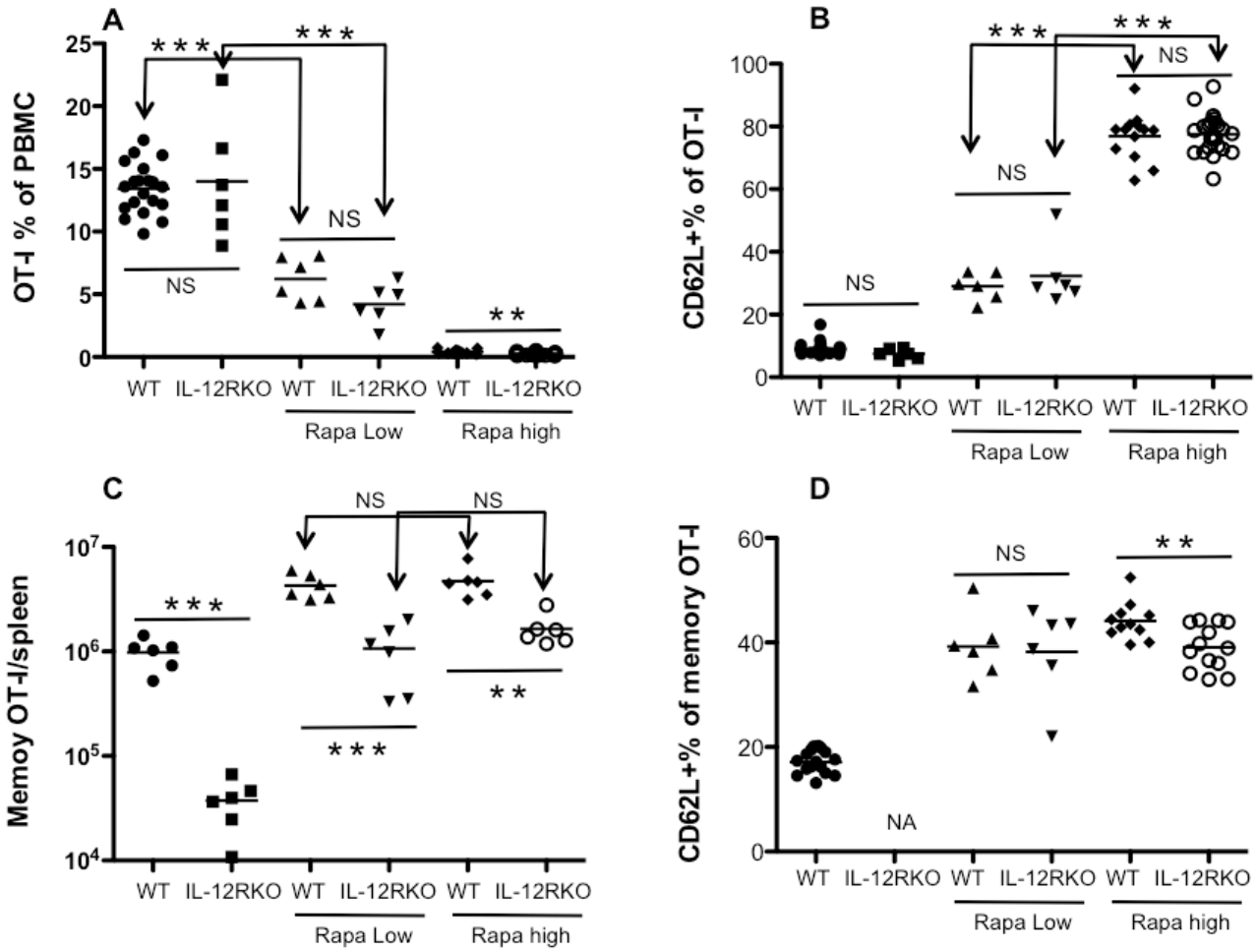


Figure 15. Long-term administration of low doses of rapamycin enhances memory CTLs to levels comparable to high doses. Naive WT or IL-12RKO OT-I cells were transferred into recipient B6 mice, which were infected with VV-OVA the next day. Low doses of rapamycin were administered daily between D−1 and 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-Is in the spleen at day 40 (c) after VV-OVA infection. (b, d) Comparison of expression of CD62L in OT-Is 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).

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-Is 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-Is were significantly lower than WT regardless of the dosage used during primary activation (Figure 16a), and expansion was only detectable 5 days after re-challenge (data not shown). Consistently, resultant secondary memory CTLs were abolished in IL-12RKO OT-Is derived from both high and low doses of rapamycin (Figure 16b). 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.

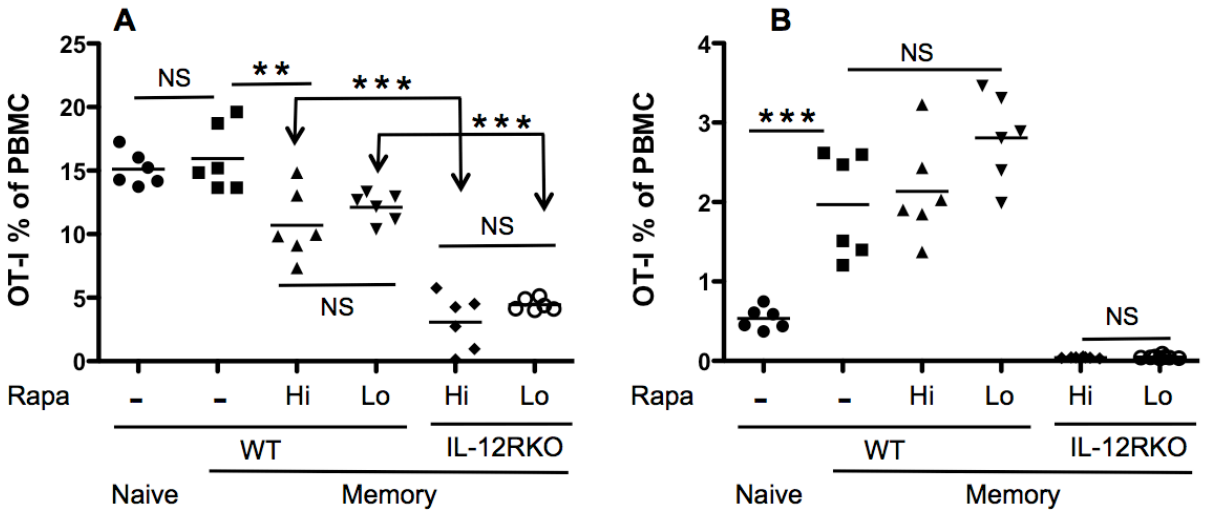


Figure 16. Requirement of the IL-12 signal for memory expansion is independent of the rapamycin dosage. Naive mice, having received naive or IL-12RKO OT-I cells, were infected with VV-OVA with high or low doses of rapamycin. Splenocytes containing 10^5 memory OT-I cells from each of the treatments were 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.

Chapter 4: Discussion[iii]

Inhibiting mTOR by rapamycin effectively enhances memory CTLs in LCMV and Listeria infections (17, 39). 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 (41), IL-12 signal is required for memory formation. Deficiency of the IL-12 signal led to almost undetectable memory, despite similar effector expansion (Figure 6a). When rapamycin was administered to recipients, memory CTLs increased (Figure 6a). 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 (57, 58), 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 6b) and subsequently improved memory CTL formation (Figure 6a). Consistent with a recent report from Ahmed *et al.* (17), high doses of rapamycin inhibited effector expansion (Figure 6a). However, this strong inhibition did not abolish expansion—CTLs still expanded substantially when high doses of rapamycin were administered (Figure 6a).

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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 6a). Compared with long-term administration of low doses of rapamycin, high doses yielded a similar effect within a shorter time window (Figure 15c).

Rapamycin promotes a central memory phenotype in a monkey model (17) and can program memory CTLs in short-term culture *in vitro* in the presence of IL-12 (14, 38). 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 (12, 59-62). Furthermore, the absence of IL-12 signal increased KLRG1 expression, an inhibitory receptor for T cells and a marker for short-lived effectors (63, 64). IL-12 marginally affected CD62L expression, if any (Figures 6d, 8d, 15b and Figure 9c). 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 6) and low doses (Figure 15). 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 (Figure 11 and 16). As a common practice in vaccination, boosting with either vectors or adjuvant is used to increase the quantity and quality of memory CTLs (42, 65-67). 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 (1).

Rapamycin may directly and indirectly regulate IL-12 signaling. IL-12R β2 expression was enhanced by rapamycin, whereas no change was observed in β1 expression during infection (Figure 13a). This could indicate that rapamycin affects IL-12 function in memory generation through differential regulation of IL-12 receptor subunits. However, both IL-12Rβ1 and β2 were inhibited by rapamycin in CTLs when IL-12 was provided *in vitro* (Figure 13c). Therefore, the enhanced expression of IL-12Rβ2 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 (14, 38), suggesting that mTOR may affect downstream IL-12 signaling. Although the IL-12 signaling was disrupted in IL-12RKO OT-Is due to β1 deficiency, the STAT4 phosphorylation was similarly upregulated by rapamycin during the early infection (Figure 13a). In addition, rapamycin enhanced STAT4 phosphorylation in CTLs *in vitro* only in the presence of IL-12 (Figure 13c), suggesting that this may be due to the combined effects of IL-12 and other cytokines, such as type I IFN (51-53, 68) and IL-3, IL-5 and IL-6 (69-76). 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-Is 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 (1). Different pathogens may cause distinct inflammatory milieus, and the induction of memory CTLs depends on unique cytokines, such as type I IFN for LCMV (77) and IL-12 for VV and LM (1, 41). The ability of CTLs to undergo secondary expansion requires the presence of pathogen-specific third-signal cytokines during priming (1). 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 (78, 79). This implies that co-stimulation and/or inflammation is essentially involved in the reactivation of memory CTLs (80). Recently, we reported that boosting with peptide requires adjuvant for memory CTL generation (42), hence it appears that cytokines are needed. The immune response to live attenuated pathogens is usually stronger than that against killed vaccines (66, 81). Thus, induction of functional memory CTLs using killed vaccines is very challenging and often requires effective adjuvants and

multiple boosts (20, 66, 81-83). 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.

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