Cutting Edge: Generation of Effector Cells That Localize to Mucosal Tissues and Form Resident Memory CD8 T Cells Is Controlled by mTOR

Ryan T. Sowell, Magdalena Rogozinska, Christine E. Nelson, Vaiva Vezys and Amanda L. Marzo

*J Immunol* 2014; 193:2067-2071; Prepublished online 28 July 2014;
doi: 10.4049/jimmunol.1400074
http://www.jimmunol.org/content/193/5/2067

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2014/07/26/jimmunol.1400074.DCSupplemental

**References**
This article cites 21 articles, 12 of which you can access for free at:
http://www.jimmunol.org/content/193/5/2067.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Cutting Edge: Generation of Effector Cells That Localize to Mucosal Tissues and Form Resident Memory CD8 T Cells Is Controlled by mTOR

Ryan T. Sowell,* Magdalena Rogozinska,* Christine E. Nelson,†‡ Vaiva Vezys,†‡ and Amanda L. Marzo*†‡

Mucosal tissues are subject to frequent pathogen exposure and are major sites for transmission of infectious disease. CD8 T cells play a critical role in controlling mucosa-acquired infections even though their migration into mucosal tissues is tightly regulated. The mechanisms and signals that control the formation of tissue-resident memory CD8 T cells are poorly understood; however, one key regulator of memory CD8 T cell differentiation, mammalian target of rapamycin kinase, can be inhibited by rapamycin. We report that, despite enhancing the formation of memory CD8 T cells in secondary lymphoid tissues, rapamycin inhibits the formation of resident memory CD8 T cells in the intestinal and vaginal mucosa. The ability of rapamycin to block the formation of functional resident CD8 T cells in mucosal tissues protected mice from a model of CD8 T cell–mediated lethal intestinal autoimmunity. These findings demonstrate an opposing role for mammalian target of rapamycin in the formation of resident versus nonresident CD8 T cell immunity. The Journal of Immunology, 2014, 193: 2067–2071.

Protective CD8 T cell immunity, in part, requires positioning memory T cells in locations of recurrent pathogen exposure. The mucosal tissues are key transmission sites for many microbes. Vaccines that favor the generation of effector memory CD8 T cells, which reside primarily in nonlymphoid compartments, were shown to protect macaques against SIV (1), presumably by maintaining a supply of resident memory CD8 T cells at the mucosal sites. Resident mucosal memory CD8 T cells are phenotypically and functionally distinct from their blood and secondary lymphoid counterparts (2, 3), of which a large population of these cells is CD103+ and constitutively expresses CD69. CD8 T cell migration and retention in the intestinal mucosa are critically dependent on the expression of gut-specific homing molecules [i.e., CCR9 (4), α4β7 (5), CD103 (6)]. Furthermore, migration to the intestine is limited to early effectors, and resident memory CD8 T cells in the small intestine (SI) do not recirculate back into secondary lymphoid tissues (7, 8). Thus, the localization of highly responsive CD8 T cells within close proximity to barriers of initial pathogen exposure could be poised to control local infections prior to dissemination through secondary lymphoid tissues. Despite this, the imprinting events necessary for CD8 T cells to localize to mucosal tissues, such as the intestine, are poorly understood.

The mammalian target of rapamycin (mTOR) is a regulator of cell proliferation, differentiation, and survival, and its activity is selectively inhibited by the drug rapamycin (9). Although originally used as an immunosuppressant in the prevention of allograft rejection, it is now understood that rapamycin also influences many facets of immune function through modulation of mTOR activity (10). In particular, mTOR was shown to regulate memory CD8 T cell differentiation (11, 12). Inhibition of mTOR by rapamycin during the priming and expansion of virus-specific CD8 T cells increases the number of memory precursors and subsequent memory CD8 T cells in secondary lymphoid tissues. However, despite generating large numbers of memory CD8 T cells in secondary lymphoid tissues, some vaccines fail to provide protective immunity (13). Given that mTOR controls the generation of memory CD8 T cells (11) and may influence T cell trafficking (10), we wanted to determine whether it has a role in the generation and maintenance of tissue-resident mucosal CD8 T cells.

*Department of Immunology and Microbiology, Rush University Medical Center, Chicago, IL 60612; †Department of Microbiology, University of Minnesota, Minneapolis, MN 55455; and ‡Center for Immunology, University of Minnesota, Minneapolis, MN 55455

Received for publication January 23, 2014. Accepted for publication July 7, 2014.

This work was supported by start-up funds from the Rush University Medical Center (to A.L.M.), a National Institutes of Health–funded program (P30 AI082151) that is supported by the following National Institutes of Health Institutes and Centers: the National Heart, Lung, and Blood Institute, and the National Center for Complementary and Alternative Medicine (to A.L.M.).
Materials and Methods

Mice and infection
Six to eight-week-old female C57BL/6J mice were purchased from the National Cancer Institute. IFABP-OVA mice were described previously (14). Mice were maintained under specific pathogen-free conditions at Rush University or the University of Minnesota in compliance with the Institutional Animal Care and Use Committee. Rapamycin was administered at a dosage of 75 μg/kg i.p. on days −1 to +7 postimmunization (unless otherwise noted) or as a control, vehicle alone (PBS + 5% DMSO) was given. Mice were immunized with 10⁶ PFU vesicular stomatitis virus (VSV)-Indiana i.v. or VSV-Indiana-OVA i.v. or with 10⁶ CFU Listeria monocytogenes–OVA (LM-OVA) orally. In adoptive-transfer experiments, 5 × 10⁵ naive OT-I cells were injected i.v., and mice were immunized 24 h later.

Isolation and analysis of Ag-specific CD8 T cells
Spleens, peripheral lymph nodes (PLNs; axillary, brachial, and inguinal were pooled), mesenteric lymph nodes (MLNs), lung, and SI lamina propria (SI LP) were harvested, and lymphocytes were isolated as previously described (2, 15). Vaginal mucosa (VM) lymphocytes were isolated from cervical vaginal tissue that was cut into small pieces and digested for 1 h with 300 U/ml type IV collagenase, followed by Percoll gradient centrifugation. In some cases, lymphocytes were stained with VSV-N (RGYVYQGL) tetramer (provided by National Institutes of Health Tetramer Facility). Cells were analyzed using a flow cytometer.

Retroviral transduction of short hairpin RNA in CD8 T cells
Activated OT-I cells were pooled from spleen and MLNs, transduced with GFP-expressing retrovirus containing short hairpin RNA (shRNA) against mTOR, and adoptively transferred into congenic recipients, as previously described (11). OT-I cells transduced with empty retrovirus were used as a control. shRNA sequences used against mTOR in CD8 T cells were described (11). OT-I cells transduced with empty retrovirus were used as a control. shRNA sequences used against mTOR in CD8 T cells were described (11). OT-I cells transduced with empty retrovirus were used as a control. shRNA sequences used against mTOR in CD8 T cells were described (11).

Statistical analysis
When means were compared between groups, error bars represent SEM. Differences (p values) between groups were calculated using an unpaired, two-tailed t test or ANOVA. Survival curves were analyzed using the log-rank test.

Results and Discussion

Rapamycin enhances memory CD8 T cell generation in lymphoid, but not mucosal, tissues
To establish whether intestinal resident memory CD8 T cell differentiation is modulated by mTOR, we used a model of acute virus infection with VSV that leads to the generation of memory CD8 T cells that accumulate in both secondary lymphoid and nonlymphoid tissues, such as the SI (16). We treated mice with a low dose of rapamycin during priming and expansion (days −1 to +7) and found no significant difference between the quantity of peripheral blood VSV-specific effector CD8 T cells generated in mice treated with rapamycin and untreated controls at day 7 (Fig. 1A). However, rapamycin increased the total number of virus-specific cells that persisted after contraction (Fig. 1A).

We then determined the numbers of VSV-specific CD8 T cells from rapamycin-treated and control mice 64 d post immunization. Consistent with our observations in the peripheral blood, rapamycin increased the quantity of memory CD8 T cells in the spleen (Fig. 1B). In contrast, rapamycin reduced the total number of virus-specific memory CD8 T cells in the SI LP. Rapamycin did not affect the formation of memory CD8 T cells in other secondary lymphoid tissues or in the vascular (“recirculating”) and tissue-resident (“non-recirculating”) memory populations within the lung (Fig. 1B, Supplemental Fig. 1A). These data show that rapamycin selectively attenuates intestinal CD8 T cell responses, indicating that mTOR plays different roles in the generation of intestinal and lymphoid memory CD8 T cells and/or their migration to the intestinal mucosa.

Rapamycin inhibits the generation of memory CD8 T cells with mucosal homing and retention markers
Expression of the integrin subunit β7, CD103, and the chemokine receptor CCR9 facilitates T cell trafficking and residence in the intestinal mucosa (4–6). To determine whether rapamycin selectively inhibits the generation of memory CD8 T cells that express gut-specific homing markers, we analyzed the expression of β7, CD103, and CCR9 on VSV-specific memory CD8 T cells. In the SI LP, rapamycin treatment decreased the frequencies of β7+, CD103+, and CCR9+ VSV-specific memory CD8 T cells compared with vehicle (Fig. 1C), suggesting that mTOR controls early signals that generate and/or maintain memory CD8 T cells that are able to traffic to and persist in the SI.

Rapamycin specifically inhibits the generation of intestinal mucosal and VM effector CD8 T cells
To determine whether rapamycin treatment inhibited the generation of effector CD8 T cells in the SI and, as a result, decreased mucosal memory, we measured the total number of VSV-specific CD8 T cells in the SI LP and MLNs 7 d after immunization. We also established the effect of rapamycin on the VM of the female reproductive tract, another mucosal tissue in which memory CD8 T cells do not recirculate. We found that the number of VSV N-tetramer+ CD8 T cells was decreased in the MLNs, SI LP, and VM in rapamycin-treated

FIGURE 1. Quantification of memory and effector VSV-specific CD8 T cells from tissues of mice treated with low-dose rapamycin (days −1 to +8) or vehicle alone. (A) Kinetics of a VSV-specific CD8 T cell response in peripheral blood. (B) Absolute number of N-Tet+ CD8 T cells in spleen, PLNs (pooled brachial, axillary, inguinal nodes), bone marrow (BM), lung, and SI LP at 64 d postimmunization. (C) Mean percentage of integrin β7+, CD103+, and CCR9 of total CD44hiN-tet+ CD8 T cells from spleen and SI LP. Results shown are from one experiment out of three, with three (A), four or five (B and C), or eight or nine (C) mice/experimental group. *p < 0.05, **p < 0.005.
mice at the peak of the virus-specific CD8 T cell response but not in the spleen or PLNs (Supplemental Fig. 1B). Rapamycin is known to enhance the formation of memory precursor effector CD8 T cells, broadly defined as being CD127hiKLRG1−, which are capable of developing into memory CD8 T cells through the modulation of the transcription factors T-bet and Eomes. Our data demonstrating reduced numbers of virus-specific CD8 T cells in the SI LP could not be explained by differences in memory precursor formation, because rapamycin enhanced the frequency of CD127hiKLRG1− cells similarly in the spleen, PLNs, and SI LP (Supplemental Fig. 1C, 1D).

To eliminate the possibility that these findings were unique to a systemic infection, we orally infected rapamycin- and vehicle-treated mice with LM-OVA. Rapamycin treatment resulted in decreased numbers of OVA-specific CD8 T cells in the MLNs and SI LP 9 d postimmunization (Supplemental Fig. 1F), as well as delayed LM-OVA clearance, with higher bacteria burdens in the spleen and SI (Supplemental Fig. 1F).

To elucidate the temporal requirements of mTOR signaling in the generation of mucosal CD8 T cells, we treated mice with rapamycin before initial T cell priming (days −1 to +6) or after priming (days 2−6), as well as during clonal expansion and migration to mucosal tissues. In these experiments we adoptively transferred a low number of naive CD45.1+ OT-I cells into congenic recipient mice that were subsequently treated with rapamycin and immunized with VSV-OVA. Using adoptive transfer of TCR-transgenic CD8 T cells that recognize a high-affinity epitope of OVA eliminates the possibility that the effect of rapamycin on mucosal CD8 T cells is related to TCR affinity differences within polyclonal Ag-specific T cells, and it controls the naive precursor frequency. Rapamycin treatment from days −1 to +6 significantly decreased the numbers of OT-I cells in the SI intraepithelial lymphocytes (SI IELs) and SI LPs at 6 d postimmunization. The number of OT-I cells isolated from rapamycin- or vehicle-treated mice from days −1 to +6 were similar in the spleen and PLNs and slightly reduced in the VM and MLNs, although the difference was not statistically significant (Fig. 2A). Rapamycin administered after priming, from day 2 to 6, inhibited the accumulation of OT-I cells in both the SI IELs and SI LP, similar to what was seen in mice treated from day −1. These data indicate that, for the generation of mucosal CD8 T cells, mTOR signaling is important during differentiation and migration to mucosal sites and not during initial T cell priming. Consistent with endogenous virus-specific CD8 T cells, we observed a downregulation of β7 and CD103 on OT-I cells isolated from the SI IELs and SI LP but few CD103 + or β7 + OT-I CD8 T cells from the VM of rapamycin-treated mice 6 d postinfec tion (Fig. 2B, Supplemental Fig. 1G). In the presence of rapamycin, fewer OT-I cells isolated from the MLNs and VM produced IFN-γ (Fig. 2B). Expression of CD103 on resident mucosal memory CD8 T cells is essential for their long-term retention; however, it is not a prerequisite for their migration into the SI. We detected decreased numbers of virus-specific CD8 T cells in the mucosal tissues as early as 4.5 d postinfec tion (data not shown), which is prior to migrating CD8 T cells’ upregulation of CD103 in the SI. Together, these data suggest that rapamycin does not directly inhibit CD103 expression; rather, the decreased CD103 expression on CD8 T cells in the SI is a consequence of rapamycin inhibiting the formation of resident mucosal memory CD8 T cell precursors. In the SI, CD103 + CD8 T cells concomitantly express CCR9 and upregulate α4β7 early after activation. However, not all α4β7 + CD8 T cells in the SI upregulate CD103 and persist as resident memory CD8 T cells. CD103 + CD8 T cells within the SI do not express KLRG1, although a significant population of CD103−α4β7−KLRG1− CD8 T cells can be found within the SI and are likely lost during the contraction phase (data not shown).

Rapamycin-treated CD8 T cells in MLNs fail to migrate to the SI

To determine the mechanism by which rapamycin blocks the formation of CD103 + resident memory CD8 T cell precursors, we examined the expression of α4β7 and CCR9 5 d postinfec tion. We found that virus-specific CD8 T cells in the MLNs and SI tended to express lower levels of α4β7 and CCR9 in rapamycin-treated mice, although the difference was not statistically significant (Supplemental Fig. 2B). The expression of CCR9 and, to a lesser degree, α4β7 is induced by retinoic acid (RA), a vitamin A metabolite that is produced by mucosal dendritic cells (17). RA is reported to activate components of the mTOR pathway (18); thus, rapamycin could block the formation of resident mucosal memory CD8 T cells through inhibiting responses to stimuli, such as RA or TGF-β, which are critical for imprinting CD8 T cells with the capacity to migrate into mucosal tissues and be retained. Furthermore, CD103 expression on CD8 T cells in the SI is dependent on TGF-β (19). We next addressed whether CD8 T cells isolated from the MLNs of rapamycin-treated mice were capable of trafficking to the SI. Five days postinfec tion,
Experiments were repeated twice with three to five mice/group. * was determined in the spleen, PLNs, lung, MLNs, SI IELs, and SI LP. **p, 2070 CUTTING EDGE: mTOR CONTROLS MUCOSAL CD8 T CELL IMMUNITY

CD8 T cell–intrinsic mTOR signals are required for the accumulation of CD8 T cells within the SI

mTOR regulates many vital cell processes; therefore, rapamycin invariably has broad effects on many cell types. To determine whether rapamycin inhibited the accumulation of CD8 T cells in the SI through either a direct effect on CD8 T cells or by indirect mechanisms, we used shRNA to target mTOR in CD8 T cells. To knock down mTOR exclusively, into congenic recipients, OT-I cells transduced with GFP-expressing retrovirus containing shRNA against mTOR. OT-I cells transduced with empty GFP-expressing retrovirus were adoptively transferred into a control group. We then assessed the percentage of GFP+ OT-I cells within the tissues 6 and 28 d postimmunization with VSV-OVA. mTOR shRNA decreased the accumulation of effector CD8 T cells overall. At 28 d postimmunization, the percentage of GFP+ OT-I cells was similar to empty retrovirus controls. However, the distribution of GFP+ OT-I cells was significantly skewed toward the lymphoid tissues in both effector and memory CD8 T cells (Fig. 3, Supplemental Fig. 2D, 2E). The lowest proportion of GFP+ OT-I cells was found within the SI IELs and SI LP, indicating that mTOR signals in CD8 T cells drive their accumulation in the SI.

FIGURE 3. shRNA knockdown of mTOR in Ag-specific CD8 T cells. OT-I cells, retrovirally transduced with either empty GFP vector (RV-Empty) or GFP vector containing mTOR shRNA (RV-mTOR shRNA), were adoptively transferred into congenic VSV-OVA–immunized recipients. At 6 (A) and 28 d (B) postimmunization, the percentage of GFP+ cells of total OT-I cells was determined in the spleen, PLNs, lung, MLNs, SI IELs, and SI LP. Experiments were repeated twice with three to five mice/group. *p < 0.05, **p < 0.005, ***p < 0.0005 one-way ANOVA. ns, not significant.

Rapamycin alters T cell responses to self-Ag expressed in the intestinal mucosa

To exclude the possibility that Ag-specific CD8 T cells were being preferentially retained in secondary lymphoid tissues, we used a system that enabled us to restrict Ag presentation to the intestinal mucosa. To do this, we used a transgenic mouse model in which OVA is expressed as a self-Ag produced in the gut epithelium (iFABP-OVA mice), and Ag draining to gut-associated lymph nodes induces robust activation of naive OT-I cells (14). We adoptively transferred OT-I cells into iFABP-OVA mice treated with rapamycin (days −1 to +5); 5 d post OT-I cell transfer, we isolated lymphocytes from various tissues, including the gut. Without the addition of inflammatory stimuli, we found increased numbers of OT-I cells in the SI LP and SI IELs compared with spleen, PLNs, MLNs, and Peyer’s patch. Rapamycin treatment reduced the numbers of OT-I cells in the SI LP and SI IELs of iFABP-OVA mice (Fig. 4A), and this corresponded with fewer CCR9+CD103+ OT-I cells within the SI IELs compared with control (Supplemental Fig. 2F). These data indicate that the effects of rapamycin are not dependent on pathogen exposure or inflammation per se. In this model, infection of iFABP-OVA mice with VSV-OVA and OT-I cell transfer results in an OVA-specific CD8 T cell–mediated destruction of the intestinal epithelium that is lethal (14, 20). To determine whether the decreased numbers of OT-I cells generated from rapamycin treatment were sufficient to prevent fatal intestinal disease, we challenged iFABP-OVA mice, which were given OT-I cells and treated with rapamycin, with VSV-OVA and monitored their survival. Rapamycin significantly increased the percentage survival of iFABP-OVA mice challenged with VSV-OVA (Fig. 4B); although epithelial damage was apparent in both groups, rapamycin treatment resulted in less blunting of the villi and shallower crypts, signifying less epithelial damage (Supplemental Fig. 2G). We propose the mechanism by which rapamycin enhanced survival is by inhibiting CD8 T cell migration and subsequent epithelial damage; thus, it could have clinical importance in the treatment of T cell–mediated intestinal autoimmune disease in which CD8 T cells ultimately infiltrate the SI, leading to destruction of the epithelium.

In summary, we show that the generation of effector and resident memory CD8 T cells in the intestinal mucosa and VM

FIGURE 4. CD8 T cell responses to self-Ag expressed exclusively in the intestinal mucosa are dampened by rapamycin. (A) Total numbers of CD45.1+ donor OT-I effector CD8 T cells in the spleen, PLNs, MLNs, Peyer’s patch (PP), SI IELs, and SI LP. (B) Survival of iFABP-OVA mice that were adoptively transferred with 5 × 106 OT-I cells, immunized with VSV-OVA, and treated (days −1 to +7) with vehicle or rapamycin (vehicle: 1/5 survivors at day 22, rapamycin: 4/4 survivors, p = 0.0073, log-rank test). Experiments were repeated twice with 3–11 mice/group. *p < 0.05. ns, not significant.
is abrogated by early low-dose rapamycin treatment, suggesting a requirement for mTOR in their development. Inhibition of mTOR activity by rapamycin during T cell proliferation and differentiation selectively disrupts the formation of effector CD8 T cell populations in the mucosal tissues, while enhancing the generation of memory CD8 T cells in secondary lymphoid tissues. We find that mTOR plays a central role in regulating the in situ induction of mucosal effector CD8 T cells, and disruption of this signal results in reduced responses to localized Ag and the ability to control a localized infection. Our results using iFABP-OVA mice whereby rapamycin decreased the generation of Ag-specific CD8 T cells in the SI, even in the absence of inflammation, eliminates the possibility that rapamycin blocks the production of, or response to, inflammatory stimuli. RA, a critical inducer of α4β7 and CCR9 expression can be produced by mucosal dendritic cells during inflammation and the steady-state. Taken together with RA’s ability to activate the mTOR pathway, our results showing mTOR-dependent induction of CCR9 and α4β7 provide a viable mechanism for rapamycin’s effect on mucosal CD8 T cells. The suggested use of rapamycin to enhance CD8 T cell responses to vaccines (21) may not provide protection against localized infections and, thus, it may not prevent the establishment of mucosa-acquired infections, such as HIV. Depending on location, Ag presentation to CD8 T cells is mediated by different dendritic cell populations, which provides a potential mechanism for tissue-specific diversity and quality of effector/memory CD8 T cell generation. A major question that remains is whether resident memory CD8 T cells within mucosal tissues are sufficient for protection against mucosal pathogen exposure or whether the recruitment of recirculating memory CD8 T cells into the site of exposure is necessary. Upon reactivation, circulating memory cells can upregulate mucosal homing markers and gain access to mucosal tissues. Thus, there is a great deal of interest in defining the contribution of resident versus recirculating memory CD8 T cells in protection from mucosal pathogens. Identifying upstream signals that augment mTOR activity may be an important therapeutic target for enhancing mucosal resident memory CD8 T cell formation and could provide insight for developing vaccine strategies that inhibit the establishment of mucosa-acquired infections.

Acknowledgments

We thank Carl Ruby for reagents, Mariana Mata and the Marzo and Veys laboratories for critical review of the manuscript, and the Rush Flow Cytometry Core supported by the James. B. Pendleton Charitable Trust.

Disclosures

The authors have no financial conflicts of interest.

References