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Cutting Edge: Rapamycin Augments Pathogen-Specific but Not Graft-Reactive CD8+ T Cell Responses

Ivana R. Ferrer,*† Maylene E. Wagener,*† Jennifer M. Robertson,*† Alexa P. Turner,*‡ Koichi Araki,*§ RAFI Ahmed,*§ Allan D. Kirk,*† Christian P. Larsen,*† and Mandy L. Ford*†

Recent evidence demonstrating that exposure to rapamycin during viral infection increased the quantity and quality of Ag-specific T cells poses an intriguing paradox, because rapamycin is used in transplantation to dampen, rather than enhance, donor-reactive T cell responses. In this report, we compared the effects of rapamycin on the Ag-specific T cell response to a bacterial infection versus a transplant. Using a transgenic system in which the Ag and the responding T cell population were identical in both cases, we observed that treatment with rapamycin augmented the Ag-specific T cell response to a pathogen, whereas it failed to do so when the Ag was presented in the context of a transplant. These results suggest that the environment in which an Ag is presented alters the influence of rapamycin on Ag-specific T cell expansion and highlights a fundamental difference between Ag presented by an infectious agent as compared with an allograft. The Journal of Immunology, 2010, 185: 000–000.

Transplantation is a life-saving treatment option for many forms of end-stage organ disease. The advent of new immunosuppressive agents over the last 30 y has dramatically increased the graft survival of almost all types of organ and tissue transplantation. One such agent, rapamycin, was isolated in the early 1970s from Streptomyces hygroscopicus and was found to potently inhibit cell proliferation and therefore possess immunosuppressive effects (1). Despite its current widespread use for the prevention of kidney allograft rejection (2), the precise effects of rapamycin on different cell types involved in rejection, including effector T cells, dendritic cells (DCs), and regulatory T cells is an area of intense investigation (1). Rapamycin exerts its effect by targeting the mammalian target of rapamycin (mTOR) (3), a serine/threonine protein kinase that has a pervasive role in many aspects of both the innate and adaptive immune response (1). Several studies exist to suggest that blockade of mTOR by rapamycin retards DC maturation and inhibits Ag uptake and presentation by DCs (4) and also attenuates T cell proliferation by inhibiting the G1→S transition. Furthermore, rapamycin has been shown by many groups to enhance the generation and function of regulatory T cells (5, 6), potentially further promoting its immunosuppressive effects during transplantation.

Recent studies in virally infected mice that had been treated with rapamycin revealed surprising and as-yet-unappreciated effects on the expansion and retention of viral-specific CD8+ T cells. Specifically, Araki et al. (7) measured the Ag-specific CD8+ T cell response following infection of mice infected with lymphocytic choriomeningitis virus in the presence or absence of rapamycin. In contrast to the expected result based on rapamycin’s known function as an immunosuppressant, this study revealed that treatment with rapamycin instead increased the quantity and quality of virus-specific memory T cells. Treatment of both mice and rhesus macaques with rapamycin resulted in an increased response to live virus or vaccination, respectively. Using RNA interference knockdown of mTOR, the regulatory-associated protein of mTOR, or the rapamycin-binding protein FKB12, these studies also demonstrated that inhibition of mTOR functioned in a T cell-intrinsic manner to enhance the quantity and quality of Ag-specific T cells (7).

Because this drug is used in many clinical transplantation immunosuppressive regimens (2), we sought to address whether treatment with rapamycin resulted in an increase in the T cell response to additional types of pathogens, such as bacterial infection, and also to study the effect of rapamycin on the expansion and retention of donor-reactive CD8+ T cells following transplantation. In short, we sought to compare the effect of rapamycin on the CD8+ T cell response to a pathogen to its effect on the CD8+ T cell response to a transplant. To accomplish this, we used an OT-I TCR transgenic system in which the same monoclonal TCR transgenic T cells responded to a bacterial pathogen infection or a skin graft. OT-I T cells recognized an epitope (SIINFEKL/Kb) that was expressed by both Listeria monocytogenes (OVA-expressing L. monocytogenes [LM-OVA]) (8) and by donor skin under the control of the β-actin promoter (9). In comparing the CD8+ T cell responses by identical monoclonal cell populations to the same epitope in the setting of either pathogen infection or transplantation, we found that treatment with rapamycin resulted in very disparate effects on
the Ag-specific cell populations. Whereas rapamycin augmented the Ag-specific CD8⁺ T cell response to a bacterium, it failed to do so in response to a transplant.

Materials and Methods

Mice

Adult male 6–8-wk-old C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). TCR transgenic OT-I and OT-II mice were purchased from Taconic Farms (Germantown, NY) and bred onto the Thy1.1⁺ background. Act-mOVA mice were produced by Dr. Marc Jenkins (University of Minnesota, Minneapolis, MN) (9). Animals received humane care and treatment in accordance with Emory University Institutional Animal Care and Use Committee guidelines (Atlanta, GA).

T cell adoptive transfers

OT-I and OT-II Thy1.1⁺ TCR transgenic T cells were harvested from spleen. The frequency of OT-I or OT-II T cells was determined prior to adoptive transfer by staining with anti-Vα2 (used by both TCRs) and anti-CD8 or anti-CD4, respectively (BD Pharmingen, San Diego, CA).

Listeria infection and rapamycin treatment

Forty-eight hours prior to Listeria infection, naïve B6 mice received an i.v. injection of 10⁵ OT-I T cells. Similar results were observed if mice received both OT-I and OT-II T cells (data not shown). Mice were then infected with 10⁴ CFU LM-OVA (8) gene i.p. on day 0. Where indicated, mice were treated with 1.5 µg/d rapamycin (Rapamune, Wyeth Pharmaceuticals, Madison, NJ) on days 0–10 postinfection or posttransplant as previously described (7).

Skin grafting

Forty-eight hours prior to membrane-bound OVA (mOVA) skin grafting, mice received an i.v. injection of 10⁶ OT-I and OT-II T cells to mimic the higher precursor frequencies observed in allospecific immune responses (10). Skin grafts (~1 cm²) were transplanted onto the dorsal thorax of recipient mice and secured with an adhesive bandage for 5 d. Rejection was defined as 90% loss of viable epidermal tissue.

Flow cytometric analyses for frequency and absolute number

Splenocytes were stained with Thy1.1-PerCP, CD8-Pacific Orange, and CD4-Pacific Blue (BD Pharmingen) for flow cytometric analysis on a BD LSRII flow cytometer (BD Biosciences, San Jose, CA). Absolute numbers of Ag-specific T cells were determined by TruCount Bead Analysis (BD Pharmingen). Data were analyzed using FlowJo Software (Tree Star, San Carlos, CA).

Intracellular cytokine staining

Cells were incubated for 4 h with 10 nM OVA257–264 (SIINFEKL) and 10 µg/ml brefeldin A (BDPharmingen) and processed using an intracellular staining kit (BD Pharmingen).

Statistical analyses

Groups were compared by Mann-Whitney nonparametric test (GraphPad Prism Software, GraphPad, La Jolla, CA).

Results

Rapamycin treatment resulted in increased Ag-specific CD8⁺ effectors following infection with OVA-expressing L. monocytogenes

In order to assess the effects of treatment with rapamycin on the Ag-specific CD8⁺ T cell response following a bacterial infection, we adoptively transferred Thy1.1⁺ OT-I T cells specific for OVA257–264/Kb into naive B6 recipients, which were then infected with 10⁴ CFU LM-OVA in the presence or absence of rapamycin. Rapamycin was administered i.p. in 500 µl sterile PBS from days 0–10 postinfection. Analysis of splenocytes indicated that the day 10 frequency (A and B) or percentage of CD62Lhi (C) of the donor-reactive CD8⁺ population was significantly increased in the rapamycin-treated recipients (p < 0.005). D–F, A total of 10⁵ Thy1.1⁺ OT-I and CD4⁺ OT-II T cells was adoptively transferred into B6 recipients 2 d prior to receiving an OVA-expressing skin graft in the presence or absence of rapamycin. Analysis of splenocytes indicated that the day 10 frequency (D and E) or percentage of CD62Lhi (F) of the donor-reactive CD8⁺ population was not increased in the rapamycin-treated recipients. Results are cumulative analyses of three independent experiments with five mice per treatment group. *p < 0.005.

FIGURE 1. Rapamycin enhanced the Ag-specific T cell response to OVA in the context of a bacterial infection but not a transplant. A–C, 10⁵ Thy1.1⁺ OT-I T cells were adoptively transferred into naive B6 recipients, which were then infected with 10⁴ CFU OVA-expressing LM-OVA. Rapamycin was administered i.p. in 500 µl sterile PBS from days 0–10 postinfection. Analysis of splenocytes indicated that the day 10 frequency (A and B) or percentage of CD62Lhi (C) of the donor-reactive CD8⁺ population was significantly increased in the rapamycin-treated recipients (p < 0.005). D–F, A total of 10⁵ Thy1.1⁺ OT-I and CD4⁺ OT-II T cells was adoptively transferred into B6 recipients 2 d prior to receiving an OVA-expressing skin graft in the presence or absence of rapamycin. Analysis of splenocytes indicated that the day 10 frequency (D and E) or percentage of CD62Lhi (F) of the donor-reactive CD8⁺ population was not increased in the rapamycin-treated recipients. Results are cumulative analyses of three independent experiments with five mice per treatment group. *p < 0.005.

1.8%, respectively; p = 0.001) (Fig. 1B). Longitudinal analysis revealed that the enhancing effects of rapamycin were observed at all time points, including memory, indicating that these observations were not simply due to altered kinetics of expansion of the Ag-specific CD8⁺ T cell response (data not shown). Consistent with recently published results in a viral infection model (7), these data indicated that treatment with rapamycin enhanced the Ag-specific T cell response following bacterial infection.

Rapamycin treatment did not result in increased donor-reactive CD8⁺ T cell responses following skin transplantation

In order to determine whether rapamycin resulted in an increased frequency of donor-reactive memory T cells following transplantation, we used the same transgenic mouse model to identify and track T cells responding to the graft in the
Rapamycin altered CD62L expression on CD8+ T cells responding to a pathogen but not a graft

In addition to enhancing the quantity of Ag-specific T cells following *Listeria* infection, we found that treatment with rapamycin also altered the quality of the Ag-specific CD8+ T cell response. CD62L has been used as a marker to differentiate between effector and memory T cells at memory time points (11). Central memory T cells (CD62Lhi) possess increased proliferative capacity and increased ability to mount a secondary response as compared with CD62LLo cells. Therefore, the presence of more CD62Lhi cells in the rapamycin-treated recipients may signify an increase in the quality of these memory T cells. Specifically, treatment with rapamycin during *Listeria* infection resulted in an increased frequency of CD62Lhi Thy1.1+ cells (17.6 ± 1.6% CD62Lhi versus 10.8 ± 1.1% in untreated controls; p = 0.0046; Fig. 1C). These results indicated that in addition to augmenting the frequency of pathogen-specific T cells, rapamycin also impacted the degree of differentiation of these cells. Therefore, we next sought to address whether treatment with rapamycin resulted in increased CD62L expression in donor-reactive CD8+ T cells stimulated by a skin graft. In contrast to our observations in the bacterial infection model, we found that splenocytes analyzed on day 10 posttransplant in rapamycin-treated recipients did not demonstrate an increase in CD62L expression (Fig. 1A).

Rapamycin differentially impacted the absolute number of IFN-γ-secreting cells generated in response to LM-OVA versus an mOVA skin graft

As shown in Fig. 1, we observed increased quantity and quality of Ag-specific CD8+ T cell populations in response to a bacterial infection following treatment with rapamycin. To further assess the quality of Ag-specific T cell responses generated against a pathogen versus a graft in the presence of rapamycin, we examined the ability of these Ag-specific T cells to produce cytokines following ex vivo restimulation (Fig. 2). Following ex vivo restimulation with OVA peptide, CD8+ T cells derived from splenocytes of rapamycin-treated LM-OVA-infected recipients exhibited an increased absolute number of IFN-γ-secreting T cells as compared with untreated and treated recipients (data not shown). Therefore, these results suggest an intrinsic difference in the effect of rapamycin on the T cell response to a graft versus a pathogen.

**FIGURE 2.** Rapamycin altered IFN-γ production in Ag-specific T cell populations following pathogen infection. A, Splenocytes from LM-OVA–infected mice, which were either left untreated or treated with rapamycin were restimulated with OVA peptide and stained for the presence of IFN-γ or TNF. B, Treatment with rapamycin resulted in an increase in the number of total IFN-γ+ T cells. C, Splenocytes from mOVA-grafted mice, which were untreated or treated with rapamycin, were restimulated with OVA peptide and stained for the presence of IFN-γ or TNF. D, Treatment with rapamycin did not result in an increase in the number of total IFN-γ+ T cells. Experiments were performed three times independently with five mice per group.
Increased IFN-γ production was observed in the rapamycin-treated recipients (Fig. A). Analysis of splenocytes indicated that the frequency of donor-reactive CD8+ population was not increased in the rapamycin-treated recipients (A). Absolute numbers of IFN-γ-producing Thy1.1+ cells were calculated by TruCount analysis (C). Experiments were performed three times independently with five mice per group. *p < 0.01.

FIGURE 3. Simultaneous infection and transplantation did not result in increased donor-specific immunity in rapamycin-treated recipients. A total of 10^6 Thy1.1+ OVA-specific CD8+ OT-I and CD4+OT-II T cells was adoptively transferred into B6 recipients. Recipients were infected with 10^6 CFU wild-type Listeria in the presence or absence of rapamycin. Analysis of splenocytes indicated that the frequency of donor-reactive CD8+ population was not increased in the rapamycin-treated recipients (A and B). Absolute numbers of IFN-γ-producing Thy1.1+ cells were calculated by TruCount analysis (C). Experiments were performed three times independently with five mice per group. *p < 0.01.

Discussion
Because rapamycin was widely appreciated to attenuate immunologic reactivity among mechanisms as altered DC differentiation and increased regulatory T cell populations (13, 14), recent evidence demonstrating that exposure to rapamycin during the course of an immune response to viral infection increased the quantity and quality of Ag-specific T cells (7) poses an intriguing paradox for the field of immunology. In this report, we have attempted to reconcile these seemingly disparate findings by directly comparing the effects of rapamycin on an Ag-specific T cell response to a bacterial infection versus a skin graft. By employing a transgenic system in which both the Ag of interest and the responding monoclonal T cell population were identical in both models, we observed that treatment with rapamycin augmented the Ag-specific T cell response to bacterial infection, whereas failing to do so when the same Ag was presented in the context of a transplant. These results suggest a fundamental difference in the effects of rapamycin on the expansion of T cell populations in response to an infectious agent as compared with an allograft. They also serve to mitigate concern that treatment with rapamycin might paradoxically augment donor-reactive T cell responses.

In addition to its known role as a signaling component downstream of the TCR/CD3 complex, mTOR has also been shown to participate in the signaling cascade downstream of many TLRs (12). Therefore, we speculated that the observed difference in the effects of rapamycin on pathogen- and donor-reactive T cell response might be due to engagement of TLR/innate immune pathways during the bacterial infection, either on APCs (4, 13, 14) or on the T cells themselves (15). These pathways would presumably not be engaged following transplantation of a skin graft. However, results indicated that infection with non-OVA–expressing Listeria concurrently with transplantation of mOVA skin grafts did not result in augmentation of anti-OVA T cell responses. One potential caveat of this experiment might be the question of whether the Listeria present in the same local environment as the graft-specific T cells during priming to influence the effect of rapamycin on these cells. However, Chong and colleagues (16) have demonstrated that Listeria infection concurrent with transplantation resulted in the prevention of tolerance induction, signifying that L. monocytogenes infection derived inflammatory signals are likely to reach graft-specific T cells in this model and that the presence of a concurrent bacterial infection and engagement of innate immune mechanisms does influence the quality of antidonor T cell responses. Importantly, however, our results indicate that this effect was not impacted by the presence or absence of rapamycin. If activation of innate immune mechanisms is not responsible, what other factors could account for the observed discrepancy of the effect of rapamycin on pathogen-specific versus graft-specific T cell responses? One possibility is that duration of Ag presentation may modulate the impact of rapamycin on Ag-specific T cell responses. Further experiments are required to test this hypothesis.

In addition, these data may have relevance to the issue of heterologous alloreactivity. Our group and others (16–18) have shown that prior or concurrent pathogen infection can augment the allospecific T cell response and alter the outcome of an allograft. This has been attributed in part to molecular mimicry (19), bystander activation (16), and other reasonably simple mechanisms. It would appear from our results that in the presence of rapamycin, presentation of the same Ag delivered either by a graft or a pathogen to the same T cell could evoke markedly different responses. As such, the outcome of stimulation with a cross-reactive Ag cannot be assumed to adversely affect allograft survival. Moreover, the order of presentation (pathogen or allograft first) and presence of immunosuppression at the time of initial presentation may significantly influence the ultimate outcome.

In summation, these results highlight disparate effects of rapamycin on T cell responses to pathogens and donor tissue and underscore the fact that there are many facets to the mTOR signaling pathway in immune cells that are still poorly understood. Still, the fact that previous studies and data presented in this study indicate that rapamycin might paradoxically enhance the Ag-specific CD8+ T cell response to viral or bacterial pathogens suggests that treatment of transplant recipients with rapamycin monotherapy may simultaneously increase immunity to a virus or vaccine (7) while inhibiting the response to an allograft. The impact of rapamycin on pathogen-specific CD8+
T cell responses in the context of other immunosuppressive agents is an important area of future research.

Disclosures
The authors have no financial conflicts of interest.

References