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Differential regulation of simultaneous anti-tumor and alloreactive CD8+ T cell responses in the same host by rapamycin

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Abstract

Rapamycin is an immunosuppressive agent routinely used in organ transplantation, but also paradoxically, exerts antiviral and anti-tumor activities. Pathogen-specific memory CD8$^+$ T cell (T_{CD8}) responses were recently found to be augmented by rapamycin. However, whether rapamycin influences the magnitude and quality of anticancer T_{CD8} responses is unknown. Importantly, how rapamycin may regulate simultaneous virus/tumor-specific and alloreactive T_{CD8} in the same host remains unexplored. To answer these questions, we primed wild-type mice with allogeneic cells concomitantly expressing simian virus 40 large tumor antigen (T Ag), a viral oncoprotein with well-defined epitopes. Rapamycin selectively enhanced the cross-priming of T_{CD8} specific for T Ag's most immunodominant epitope called site IV but not T_{CD8} alloreactivity. Rapamycin-treated mice also had a high percentage of splenic CD127^{high}KLRG1^{low} T_{CD8} as well as an increased frequency of site IV-specific T cells long after the peak of their primary response. When site IV was presented as a cytosolic minigene encoded by a recombinant vaccinia virus, rapamycin failed to boost the site IV-specific response. Therefore, the nature and presentation mode of antigen determine the susceptibility to the adjuvant effect of rapamycin. Our findings reveal the unexpected benefit of rapamycin treatment in recipients of allografts co-expressing tumor/viral Ags.

Keywords

Rapamycin; mTOR; CD8$^+$ T cells; alloreactivity; anti-tumor response; memory

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Disclosures

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

Supporting Information

Additional supporting information may be found in the online version of this article.

Supplemental Figure 1 depicts a head-to-head comparison of ICS for IFN-γ and tetramer staining for detection of site I- and site IV-specific T_{CD8} at the peak of their primary response.

Supplemental Figure 2 demonstrates the effect of continuous rapamycin treatment on long-term frequencies of T Ag-specific and alloreactive T_{CD8}.

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Introduction

Allograft rejection by immunological mechanisms constitutes a formidable obstacle to life-saving organ transplantation. Initially discovered as an anti-fungal macrolide produced by *Streptomyces hygroscopicus*, rapamycin is an immunosuppressive agent commonly used in the clinic to hamper alloaggressive T cells in renal graft recipients (1). It potently and specifically inhibits the mammalian target of rapamycin (mTOR), an intracellular serine/threonine protein kinase that controls cellular metabolism, growth and survival. Various aspects of innate and adaptive immune responses are modulated by mTOR (2). The inhibition of the mTOR signaling pathway by rapamycin leads to altered T cell trafficking (3), attenuated effector T cell proliferation and enhanced regulatory T cell function (4,5), all of which likely contribute to rapamycin-induced immunosuppression.

Recent studies have revealed that rapamycin surprisingly improves, rather than weakens, memory $CD8^+$ T cell (T_{CD8}) responses to lymphocytic choriomeningitis virus (LCMV) in mice (6) and vaccinia virus (VV) in rhesus macaques (6,7). However, several important questions remain regarding T_{CD8} immunomodulation by rapamycin. First, it is not clear whether T_{CD8} specific for tumor antigens (Ags) are controlled by mTOR. This is particularly important given the increased risk of malignancy in allograft recipients. Second, to what degree rapamycin treatment influences T_{CD8} cross-priming is unknown. Cross-priming is spearheaded by professional Ag-presenting cells (pAPCs), particularly dendritic cells (DCs), which acquire antigenic materials from client cells (e.g., an allogeneic graft cell) that are incapable of activating naïve T_{CD8} on their own (8). Cross-priming is a robust pathway for inducing T_{CD8} responses to tumors of non-hematopoietic origin and to viruses that paralyze the MHC class I pathway in infected host cells. Although Ags displayed by allografted tissues may trigger T_{CD8} cross-priming, T_{CD8} alloreactivity may also result from direct priming. Third, whether rapamycin affects the epitope breadth of T_{CD8} responses is not understood. Of thousands of potentially immunogenic peptides harbored by complex Ags, only a handful elicit detectable T_{CD8} responses of varying magnitude, thus creating an immunodominance hierarchy among Ag-specific T_{CD8} clones (9). Immunodominance may influence the effectiveness of T_{CD8} responses to tumors, pathogens and transplants. Last, but certainly not least, it is not known how rapamycin may regulate concurrent T_{CD8} responses mounted towards allografts and other Ags in the same host at the same time. This is a relevant question in light of the clinical facts that: 1) current organ deficit may justify the usage of allografts prepared from “high-risk” kidneys containing tumor masses for recipients with limited life expectancy (10), which could introduce tumor Ags to the recipients' immune system; 2) donor-derived infections with a variety of microbes (e.g., cytomegalovirus and BK polyoma virus) still occur with significant frequency (11); 3) community-acquired and nosocomial infections or reactivation of endogenous latent viruses are common in immunocompromised allograft recipients.

To address all the above questions in a non-transgenic, physiologically relevant setting, we primed wild-type mice with allogeneic kidney epithelial cells expressing a clinically relevant tumor Ag, the simian virus 40 (SV40) large tumor Ag (T Ag), which is in fact homologous to the BK virus T Ag detected in human kidneys. We demonstrate that rapamycin selectively improves the T_{CD8} response elicited by cross-priming against the most immunodominant epitope of T Ag (site IV) while not affecting or slightly attenuating alloreactive T_{CD8} present in the same host. In addition, rapamycin failed to boost the T_{CD8} response to site IV in mice infected with a recombinant vaccinia virus (rVV) expressing site IV. Therefore, the mode of T_{CD8} priming and the immunodominance status of targeted epitopes determine susceptibility to the immunostimulatory effect of rapamycin on T_{CD8}. Our findings have clear clinical implications in allotransplantation and in therapeutic vaccine design.

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Materials and Methods

Mice

Adult female C57BL/6 (B6)(H-2^b) mice were purchased from Charles River Canada Inc. (St. Constant, QC), housed at the University of Western Ontario animal care facility under specific pathogen-free conditions, and cared for in accordance with institutional and national guidelines.

Cell lines

The SV40-transformed cell lines KD2SV (H-2^d) and C57SV (H-2^b) were grown in Dulbecco modified Eagle medium supplemented with 5% fetal bovine serum (FBS). The mouse mastocytoma cell line P815 (H-2^d) was maintained in complete RPMI 1640 medium containing 10% FBS, nonessential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate and 50 μM 2-mercaptoethanol.

Immunization and rapamycin treatment

Age- and gender-matched mice received daily intraperitoneal (*i.p.*) injections of freshly prepared rapamycin (LC Laboratories, Woburn, MA) at 1.5 μg/dose in PBS or of vehicle (PBS containing Phosal 50 PG and Tween 80). This regimen provides a blood rapamycin concentration of ~5–20 ng/ml (6), which is consistent with its clinical dosing in humans. Treatment with rapamycin or vehicle started one day prior to *i.p.* immunization with 2×10^7 allogeneic KD2SV cells or 5×10^6 plaque-forming units of a rVV expressing the T Ag's immunodominant peptide site IV (rVV-IV) and ended one day before the animals were euthanized.

Intracellular cytokine staining (ICS) and cytofluorimetric analyses

Unless otherwise indicated, mice were euthanized 9 or 7 days after immunization with KD2SV cells or rVV-IV, respectively, time points at which corresponding primary T_{CD8} responses reach their peak (12). Erythrocyte-depleted splonocytes and peritoneal exudate cells were then stimulated *ex vivo*, as appropriate, with C57SV cells, KD2SV cells, P815 cells or the following synthetic peptides corresponding to T Ag- and VV-derived epitopes (12,13): T Ag peptides: SAINNYAQKL (site I), CKGVNKEYL (site II/III), VVYDFLKC (site IV), QGINNLDNL (site V); VV peptides: TSYKFESV ($B8R_{20}$), AAFEFINSL ($A47L_{138}$), YSLPNAGDVI ($K3L_6$), YAPVSPIVI ($A42R_{88}$), VSLDYINTM ($A19L_{47}$). An H-2^b-restricted peptide derived from HSV-1, gB_{498} (SSIEFARL), served as irrelevant control. All the above peptides were >95% pure and generously provided by Drs. Jonathan Yewdell and Jack Bennink (National Institutes of Health). All peptides were used at a 500 nM final concentration. After 2-hour incubation at 37°C, brefeldin A was added at 10 μg/ml and cultures were continued for an additional 3 hours. Cells were then washed, stained for surface CD8α, CD127 (IL-7Rα), killer cell lectin-like receptor G1 (KLRG1), fixed and permeablized to enable staining for intracellular interferon (IFN)-γ and Bcl-2. All fluorochrome-labeled Abs were from eBioscience except for anti-KLRG1-FITC (Southern Biotech, Birmingham, AL) and anti-Bcl-2-FITC (BD Pharmingen). Isotype controls were purchased from eBioscience or BD Pharmingen. MHC class I tetramers were prepared and used as we previously described (14). A BD FACSCanto II flow cytometer and FlowJo software (Tree Star, Ashland, OR) were used for data acquisition and analysis. The percentage of IFN-γ^+ or tetramer$^+$ cells was determined after live gating on $CD8^+$ events and Ag-specific T_{CD8} were enumerated accordingly.

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Statistical analysis

Statistical comparisons were performed using Student's *t*-test with the aid of GraphPad Prism software. *, ** and *** denote $p<0.05$, $p<0.01$ and $p<0.001$, respectively.

Results and Discussion

mTOR regulation of concomitant tumor-specific and alloreactive T_{CD8} responses

It was recently demonstrated that anti-pathogen T_{CD8} can be augmented by rapamycin treatment (6,7,15). An important and elegant study by Ferrer et al. found that rapamycin enhances the responsiveness of adoptively transferred ovalbumin-specific T_{CD8} in mice infected with recombinant *Listeria monocytogenes* encoding ovalbumin but not in recipients of an ovalbumin-expressing skin allograft (15). The above study examined transgenic T_{CD8} responses in parallel, not in the same host. Therefore, we set to explore the effect of rapamycin on simultaneously ongoing T_{CD8} responses against tumor/viral Ags and alloantigens within the same wild-type animal. To do so, we injected B6 mice (H-2^b) with KD2SV cells (H-2^d) that are transformed with SV40 and, as such, express T Ag, a viral oncoprotein with well characterized T_{CD8} epitopes (14). T_{CD8} responses in this model mimic the "real life" situation because they are elicited against two types of clinically relevant Ags (i.e., alloantigens and T Ag) expressed by kidney epithelial cells (a known target of T cells in renal allograft recipients) in wild-type animals harboring a natural T cell repertoire.

We first confirmed that in our model, T Ag-specific and alloreactive T_{CD8} responses require *in vivo* priming with KD2SV cells and are not detectable in naïve animals (Fig. 1A). Treatment with rapamycin increased the frequency of both the splenic and peritoneal T_{CD8} specific for site IV, the most immunodominant epitope of T Ag (14), as judged by intracellular staining for IFN-γ (Fig. 1B, 1C). Peritoneal and splenic T_{CD8} represent local and systemic responders to site IV, respectively (8,12). There was also a trend for an enhanced T_{CD8} response to C57SV cells, T Ag^+ fibroblastic cells of B6 origin, when they were used in lieu of T Ag-derived peptides for *ex vivo* T_{CD8} restimulation. We found a similar increase in both the absolute number and mean fluorescence intensity (MFI) of site IV-specific IFN-γ^+ T_{CD8} in the spleens of rapamycin-treated animals (Fig. 2A, 2B). Contrary to T Ag-specific T_{CD8}, the frequency of alloreactive T_{CD8}, defined by their *ex vivo* responsiveness to KD2SV cells, was unaltered or decreased to varying degrees upon rapamycin treatment (Fig. 1). This decrease was most pronounced within the peritoneal cavity (Fig. 1C). This response is of allogeneic nature and independent of T Ag expression by KD2SV cells because these cells express H-2^d allomorphs and cannot be directly recognized by T Ag-specific T_{CD8} that are H-2^b-restricted. The above notion is supported by our observation that rapamycin treatment of KD2SV-primed mice failed to increase the frequency of alloreactive cells restimulated with P815 cells, a T Ag^- H-2^{d+} cell line (Fig. 1B, 1C).

Our finding that site IV-specific T_{CD8} in rapamycin-treated mice produce more IFN-γ on a per cell basis – hence their higher MFI – indicates that rapamycin improves the functional fitness of these T_{CD8}. This is consistent with our unpublished observation that treatment with rapamycin also amplifies cytotoxic responses of cross-primed, T Ag-specific T_{CD8} (data not shown).

Previous studies have documented the positive effect of rapamycin on memory but not primary T_{CD8} responses. Rapamycin treatment reportedly failed to increase LCMV-specific T_{CD8} numbers at the peak of their primary response (6). In contrast, the primary T_{CD8} response to VV in rhesus macaques and that to a heat shock protein-based vaccine in mice were boosted by the inhibition of mTOR (7,16). These discrepancies may have stemmed

from different readouts used in these studies. The former study enumerated LCMV-specific T_{CD8} by tetramer staining, whereas the latter two studies used functional assays similar to ours. In fact, when we detected site IV-specific T_{CD8} by tetramer staining in a head-to-head comparison with ICS, we did not find any difference between mice receiving rapamycin or vehicle by tetramer staining (supplemental Fig. 1).

It was of interest to determine whether rapamycin affects the quality of primary T Ag-specific T_{CD8} and their progression to a memory state. We found that the site IV-specific T_{CD8} pool in rapamycin-treated animals had a higher proportion of $CD127^{high}KLRG1^{low}$ cells and a lower proportion of $CD127^{low}KLRG1^{high}$ cells, which are considered memory T_{CD8} precursors and short-lived effectors, respectively (6)(Fig. 3A, 3B). Rapamycin treatment also increased the expression of the pro-survival protein Bcl-2 in site IV-specific T_{CD8} at the peak of their primary response (Fig. 3C). Importantly, treatment with rapamycin during the initial priming phase (i.e., during the first 9 days) led to a higher frequency of site IV-specific T_{CD8} detected at a later time point (day 27)(Fig. 3D). In a different setting that simulates clinical conditions requiring continuous treatment, daily administration of rapamycin up until day 27 resulted in a higher proportion of T Ag-specific (but not alloreactive) T_{CD8} (supplemental Fig. 2). These results collectively show that rapamycin ameliorates the functional fitness of primary anti-tumor T_{CD8} and raises both their primary and long-term frequencies.

Inhibition of mTOR affects T_{CD8} cross-priming and immunodominance

The T Ag-specific response in our model occurs exclusively through cross-priming (8,12). This is because: 1) KD2SV cells are of kidney epithelial origin, not pAPCs, and lack B7 costimulatory molecules, a prerequisite for naïve T_{CD8} activation; 2) they are allogeneic to B6 mice and unable to directly prime T_{CD8} in this strain according to the rule of MHC restriction; 3) they are transformed with subgenomic fragments of SV40 and fail to produce SV40 virions, thus eliminating any possibility that the ensuing T_{CD8} responses are due to the infection of host pAPCs (8). Therefore, our finding that the T_{CD8} response to T Ag in this model is improved by rapamycin constitutes the initial report describing the effect of this agent on cross-priming. This is important for allotransplantation because T_{CD8} responses to microbial and tumor Ags of donor origin, which are believed to occur at least partially through cross-priming, are likely to be heightened by rapamycin. We have recently found that anti-influenza T_{CD8} responses can also be augmented by rapamycin in a cross-priming model (8)(unpublished data).

We found that rapamycin strengthens the T_{CD8} response to site IV, but typically not those targeting subdominant epitopes (sites I, II/III and V). This indicates that even among T_{CD8} clones recognizing the same Ag, some are more prone than others to the immunostimulatory effect of rapamycin. We previously reported that site IV-specific T_{CD8} are sufficient for eradication of T Ag-induced choroid plexus brain tumors in irradiated mice (17). However, it is noteworthy that T_{CD8} clones specific for immunodominant epitopes are not always necessarily the most protective T_{CD8} against all forms of cancer and infectious diseases. Therefore, the selective adjuvanticity of rapamycin for some but not all T_{CD8} clones need to be taken into consideration in therapeutic vaccine design.

Mode of Ag presentation determines the susceptibility of T_{CD8} responses to rapamycin adjuvanticity

Next, we asked whether rapamycin affects T_{CD8} responses to antigenic peptides encoded by a viral vector. We infected vehicle- and rapamycin-treated mice with a rVV that expresses site IV as a cytosolic minigene (12). Direct priming is presumed to be the predominant pathway in activating naïve T_{CD8} recognizing peptides encoded by such minigenes. This

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experiment also enabled us to examine the effect of rapamycin on mouse T_{CD8} responses to VV epitopes that we previously characterized (13). T_{CD8} responses to both site IV and the VV-derived epitopes $B8R_{20}$, $A47L_{138}$, $K3L_{6}$, $A42R_{88}$ and $A19L_{47}$ remained unaltered upon rapamycin treatment (Fig. 4 and data not shown). Therefore, we conclude that: 1) regardless of whether site IV-specific T_{CD8} activation following rVV-IV infection can be dubbed as direct priming, the mode of Ag presentation can clearly dictate the susceptibility of T_{CD8} responses to rapamycin; 2) the adjuvanticity of rapamycin cannot be generalized to all pathogen-specific T_{CD8} responses, and even to T_{CD8} responses against the same pathogen in different host species. This is because mouse T_{CD8} responses to VV epitopes are resistant to rapamycin treatment, whereas the bulk VV-specific T_{CD8} response is reportedly augmented in rapamycin-treated rhesus macaques (7). It will be important to explore the susceptibility of VV-specific T_{CD8} to rapamycin in humans since rVVs are pursued as suitable vectors in therapeutic vaccination.

How rapamycin modulates T_{CD8} responses is unclear. Using RNA interference to knock down mTOR, raptor [an important component of the mTOR complex 1(mTORC1)] or FKBP12 (a binding partner of rapamycin) exclusively in LCMV-specific transgenic T_{CD8}, a previous study found that rapamycin operates in a T cell-intrinsic fashion to accelerate memory T_{CD8} differentiation (6). Whether this is true also for wild-type T_{CD8} is currently unknown. The role of mTORC2, whose activity may be reduced in some cell types after prolonged exposure to rapamycin (18), remains to be elucidated. mTOR is known to modulate autophagy in DCs, and rapamycin-induced autophagy in these cells enhances their ability to prime T cells *in vitro* (19). We favor the possibility that APCs may participate in modulation of T_{CD8} by rapamycin. This is because: 1) tumor-specific and alloreactive T_{CD8} primed in the same host behave differently in response to rapamycin; 2) T_{CD8} clones recognizing various epitopes of the same Ag show variation in response to the immunostimulatory effect of rapamycin (site IV versus other epitopes); 3) the T_{CD8} priming route for the same epitope determines the response to rapamycin (site IV expressed by allogeneic non-APCs as opposed to site IV encoded by a rVV), potentially implicating various APC subsets in the observed effect; 4) T_{CD8} found in different environments exhibit varying degrees of susceptibility to rapamycin (splenic versus peritoneal alloresponses). The activity of mTORC1 and/or mTORC2 and the specialized functions of distinct APC subsets (e.g., autophagy) may be subject to differential rapamycin regulation. Infection with replicating viral vectors (e.g., rVV) expressing tumor Ags may yield a high Ag load and simultaneously trigger viral pattern recognition receptors within APCs. This would be absent in responses to cell-associated Ags.

In summary, we show for the first time that rapamycin augments the vigor, fitness and quality of T_{CD8} responses induced by cross-priming against a clinically relevant viral oncoprotein but not the alloreactive T_{CD8} response occurring in the same host. The ultimate question is whether human T_{CD8} are prone to the adjuvant effect of rapamycin. Rapamycin is not only used in allograft recipients but is also an approved therapeutic agent for advanced renal cell carcinoma. How T_{CD8} in allograft recipients and cancer patients under rapamycin therapy respond to viruses and tumor Ags warrants further investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

B6	C57BL/6
FBS	fetal bovine serum
ICS	intracellular cytokine staining
IFN	interferon
KLRG1	killer cell lectin-like receptor G1
LCMV	lymphocytic choriomeningitis virus
MFI	mean fluorescence intensity
mTOR	mammalian target of rapamycin
mTORC	mTOR complex
T_{CD8}	$CD8^+$ T cell
rVV	recombinant vaccinia virus
SV40	simian virus 40
T Ag	large tumor antigen

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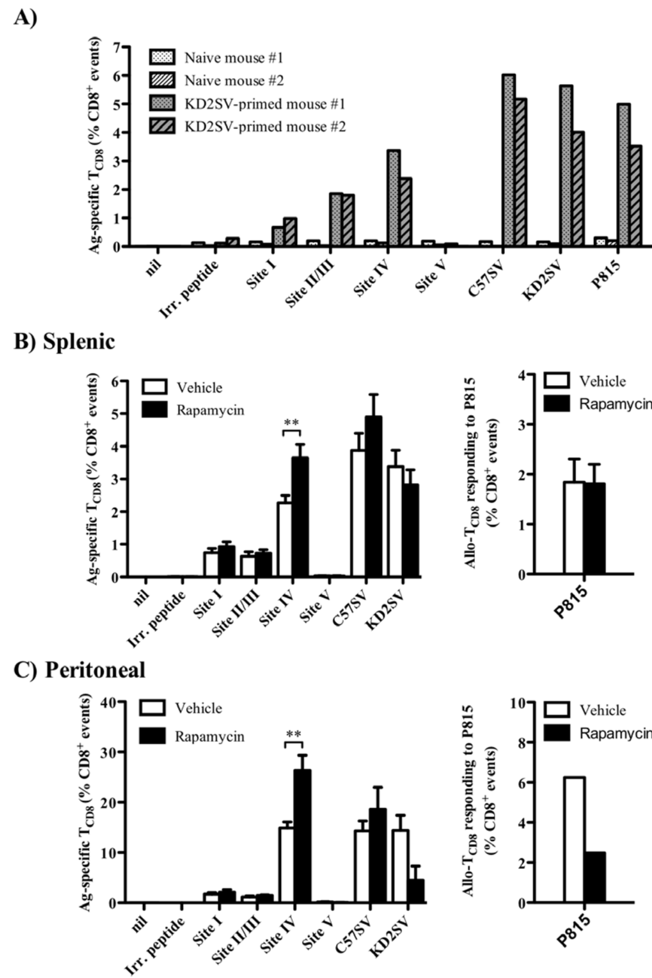

Figure 1.

Treatment with rapamycin increases the frequency of functional T_{CD8} specific for the T Ag immunodominant epitope (site IV), but not that of alloreactive T_{CD8}. To confirm the requirement for *in vivo* priming in the generation of T Ag-specific and alloreactive T_{CD8} responses, splenocytes from naïve B6 mice or B6 mice immunized with allogeneic (H-2^d) T Ag^+ KD2SV cells were restimulated *ex vivo* with T Ag-derived peptides (sites I, II/III, IV or V), syngeneic (H-2^b) T Ag^+ C57SV cells, allogeneic KD2SV cells, or allogeneic P815 cells used at 2×10^5 cells/well. The frequency of cognate T_{CD8} was calculated as the percentage of IFN-γ^+ cells after live gating on $CD8^+$ events. Individual mouse data are shown (A). To assess the effect of rapamycin, splenocytes (B) and peritoneal exudate cells (C) from vehicle- and rapamycin-treated B6 mice that were immunized with KD2SV cells were restimulated *ex vivo* with T Ag-derived peptides, C57SV, KD2SV or P815 cells. The frequency of cognate T_{CD8} was determined as described above. Data are shown as mean ± SEM obtained from 19 mice/group pooled from 5 independent experiments except in the case of the T_{CD8} response to P815 cells that was assessed in two experiments.

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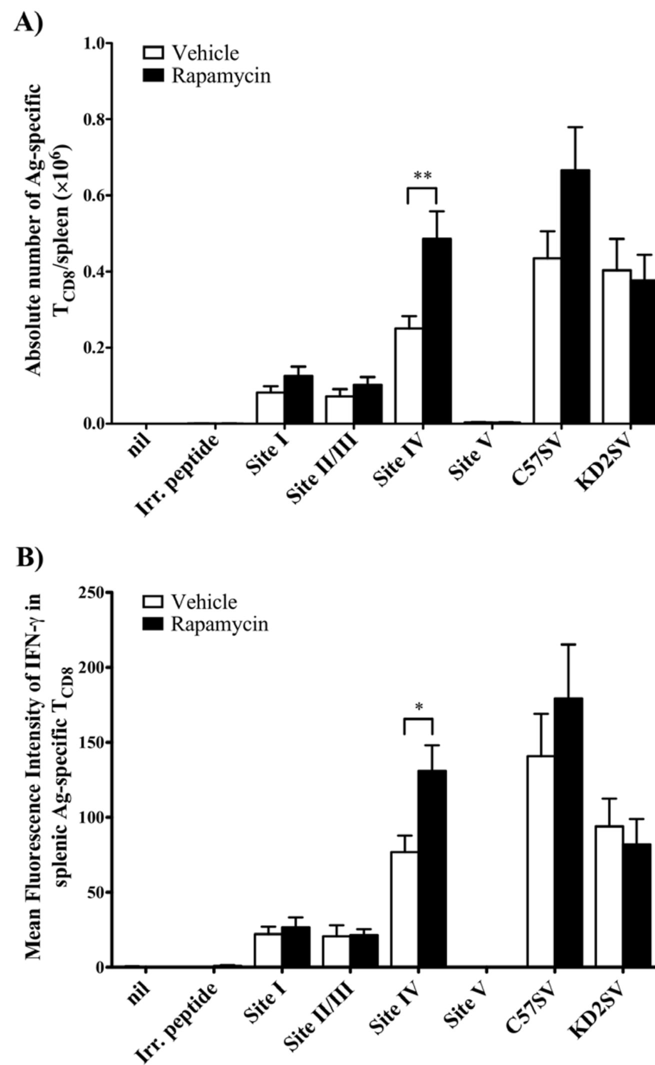

Figure 2.

Treatment with rapamycin increases the absolute number and mean IFN-γ production (on a per cell basis) of splenic site IV-specific T_{CD8}, but not those of alloreactive T_{CD8}. Splenocytes from vehicle- and rapamycin-treated B6 mice that were immunized with KD2SV cells were restimulated *ex vivo* with peptides corresponding to T Ag epitopes (sites I, II/III, IV or V), syngeneic T Ag^{+} C57SV cells, or allogeneic KD2SV cells. The frequency of cognate IFN-γ^{+} T_{CD8} was used to enumerate the absolute number of these cells within each spleen (A), and their MFI for IFN-γ was also determined using FlowJo software (B). Data are shown as mean ± SEM obtained from 19 mice/group pooled from 5 independent experiments.

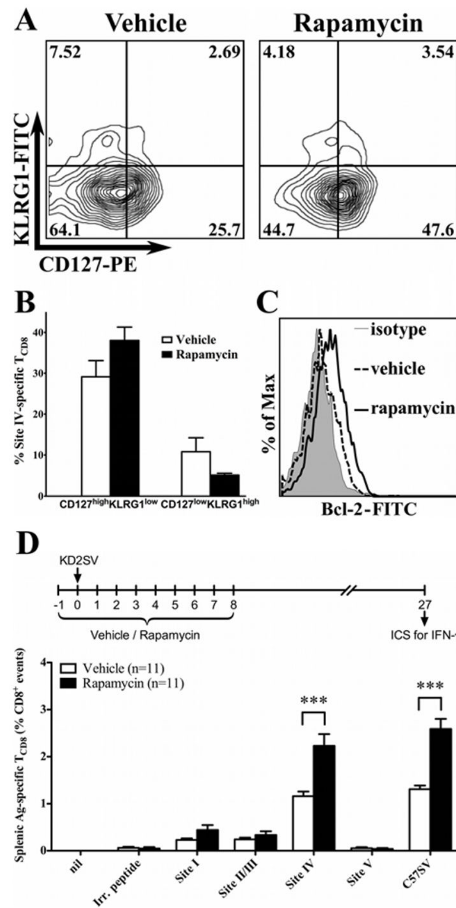

Figure 3.

Rapamycin treatment improves the quality of primary T Ag-specific T_{CD8} and promotes their progression to memory cells. Site IV-specific T_{CD8} identified by ICS for IFN-γ were gated upon and assessed for their expression of CD127 and KLRG1 (A,B) as well as intracellular Bcl-2 (C). Representative FACS plots for these markers are shown. In addition, the frequencies of $CD127^{high}KLRG1^{low}$ (memory T_{CD8} precursors) and $CD127^{low}KLRG1^{high}$ (short-lived effectors) are shown for 6 mice/group (B). Statistical comparisons revealed that rapamycin-treated mice had a higher proportion of $CD127^{high}KLRG1^{low}$ cells compared with vehicle-treated animals (38 ± 3.3 vs. 29.1 ± 4, p=0.11) and a lower proportion of $CD127^{low}KLRG1^{high}$ cells (5.1 ± 0.4 vs. 10.8 ± 3.4, p=0.15). In separate experiments, B6 mice were immunized with KD2SV cells and treated with rapamycin or vehicle during the initial priming phase as illustrated (D). Mice were left untreated until day 27, at which point the frequency of T Ag-specific T_{CD8} was determined by ICS for IFN-γ. Data are shown as mean ± SEM obtained from 11 mice/group pooled from 3 independent experiments.

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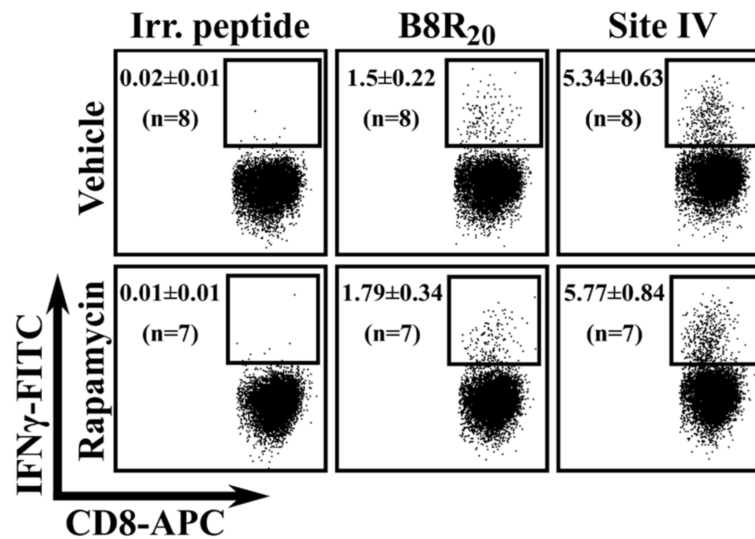

Figure 4.

VV- and site IV-specific T_{CD8} induced through infection with rVV-IV are not affected by rapamycin. Splenocytes from rVV-IV-infected mice that received daily treatment of rapamycin or vehicle were restimulated *ex vivo* with VV-derived peptides (including $B8R_{20}$) or the T Ag site IV peptide. The frequencies of epitope-specific T_{CD8} are shown as mean ± SEM obtained from 7–8 mice/group pooled from 2 independent experiments that yielded almost identical results. Representative dot plots are also shown.