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Uncoupling p70S6 Kinase Activation and Proliferation: Rapamycin-Resistant Proliferation of Human CD8+ T Lymphocytes

Jacqueline M. Slavik,* Dong-Gyun Lim,* Steven J. Burakoff, † and David A. Hafler2,∗

Rapamycin is a fungal macrolide that inhibits the proliferation of T cells. Studies in both animals and humans have found that rapamycin significantly reduces graft rejection. However, though CD8+ T cells are involved in graft infiltration and rejection, little is known regarding the effects of rapamycin on CD8+ human T cell responses. In this study, we examined the mechanism of rapamycin-induced inhibition of Ag-driven activation of CD8+ T cells. Surprisingly, a heterogeneous proliferative response in the presence of rapamycin was observed among different Ag-specific CD8+ T cell clones; this was also observed in CD8+ peripheral blood T cells activated with TCR cross-linking ex vivo. Inhibition of T cell proliferation by rapamycin was controlled by both the strength of signal delivered through the Ag receptor as well as the specific costimulatory signals received by the T cell. Rapamycin-resistant proliferation occurred despite inhibition of p70S6 kinase activity. Moreover, rapamycin-resistant proliferation of the CD8+ T cell clones was blocked by anti-IL-2 Abs, suggesting that while some of the parallel pathways triggered by IL-2R signaling are sensitive to the effects of rapamycin, others account for the Ag-driven rapamycin resistance. These data provide a new framework for examining the specific mechanism of action of rapamycin in human disease. The Journal of Immunology, 2001, 166: 3201–3209.

Rapamycin (Sirolimus) was originally identified in a screen for antibiotic activity, but instead was found to have immunosuppressant activity (1). Recognition of the structural similarity of rapamycin and FK506 led to the study of rapamycin for use in preventing organ transplant rejection (2). Rapamycin suppresses graft rejection and reduces graft vs host disease in animal models. Clinical studies in humans have shown that treatment with rapamycin has a beneficial effect (3–8). A recent study demonstrated a dramatic increase in islet cell transplant function. These studies were supported by grants to D.A.H. from the National Institutes of Health (P01 AI39671 and R01 AI44447-01), from the Juvenile Diabetes Foundation International, and from the Juvenile Diabetes Foundation International. E-mail address: dhafler@rics.bwh.harvard.edu

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Abbreviations used in this paper: mTOR, mammalian target of rapamycin; HTLV-1, human T cell leukemia virus-1; PBT, peripheral blood T cells; PVDF, polyvinylidene difluoride.
In this study, we examined the effects of rapamycin on the activation of human CD8⁺ T cells. Our studies show that proliferation of CD8⁺ T cells can be resistant to inhibition by rapamycin. This is true for both CD8⁺ primary T cells as well as for a subset of CD8⁺ human T cell clones. Rapamycin-resistant proliferation of the CD8⁺ T cell clones can be blocked by anti-IL-2 Abs, suggesting that while some of the parallel pathways triggered by IL-2R signaling are sensitive to the effects of rapamycin, others account for the Ag-driven rapamycin resistance. These data provide a new framework for examining rapamycin’s specific mechanism of action in the treatment of human disease.

Materials and Methods

Cells and cell culture

Lymphocytes were isolated from the whole blood of normal donors following platelet separation by centrifugation through Ficoll-Paque (Amer- sham, Uppsala, Switzerland) and plastic adherence. CD8⁺ T cells were positively selected following incubation with anti-human CD8 microbeads using the Dynal bead magnetic cell separation system (Dynal, Lake Success, NY). Purified CD8⁺ T cells were rested overnight at 37°C before use. CD8⁺ T cells were cultured at 37°C with 5% CO₂ in RPMI 1640 (Bio- Whitaker, Walkersville, MD) with 10% heat-inactivated FCS (Irvine Scientific, Santa Ana, CA), 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, and 2 mM L-glutamine (all obtained from BioWhittaker). CD8⁺ human T cell clones reactive to human T cell leukemia virus-1 (HTLV-1) peptide Tax11–19 were generated as described and have been extensively characterized functionally elsewhere (28). Briefly, CD8⁺/HLA-A*0201/Tax11–19 tetramer-positive T cells were isolated from the peripheral blood of an HLA-A*0201-expressing patient with typical HTLV-1 myelopathy and single cell cloned. All of the tetramer-binding T cells that were expanded in vitro proliferated in response to stimulation by HLA-A*0201/LFA-3-expressing human hamster ovary cells loaded with Tax11–19 peptide. For use in experiments, T cell clones were thawed, resuspended with PHA plus irradiated mononuclear cells, and expanded in RPMI 1640 containing 10% heat-inactivated pooled human serum (Omega Scientific, Tarzana, CA), 10% T-stim (Collaborative Biomedical Products, Bedford, MA), 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, and 2 mM L-glutamine (all obtained from BioWhittaker). The generation of the EBV-transformed B cell lines DAH (HLA-A*0205⁺) and KSB (HLA-A*0201⁺) has been described (28).

Antibodies

The murine anti-human CD28 mAb 3D10 (generous gift of Mary Colling, Genetics Institute, Cambridge, MA), the murine anti-human CD3 mAb OKT3 (American Type Culture Collection (ATCC), Manassas, VA), the anti-human OKT8 mAb (ATCC), and the anti-human CD2 OKT11 (ATCC) were purchased from ascites. The anti-p70s6k polyclonal Ab was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The neutralizing anti-human IL-15 and the anti-human IL-2 were purchased from R&D Systems (Minneapolis, MN).

Proliferation assay

Cells were pretreated with rapamycin or ethanol (vehicle control) for 30 min to 2 h before use in proliferation assays. EBV-transformed B cells or transfected Chinese hamster ovary cells were irradiated with 5000 rad or treated with mitomycin C (100 μg/ml for 2 h), respectively, pulsed with the peptide or PBS control for 2 h at 37°C, and washed twice to remove free peptide. Plates were seeded with 2 × 10⁴ APCs per well. For Ab stimulations, plates were coated with anti-CD3, or with anti-CD3 plus either anti-CD28, anti-CD8, or anti-CD2, as indicated, at 37°C for at least 2 h, then washed five times with twice the volume of PBS to remove free Ab before use. T cells were plated in 96-well round-bottom plates (Costar, Cambridge, MA) at 10⁵/well (in triplicate) in a final volume of 200 μl and cultured for 72 h. Proliferation was assessed by the incorporation of [³H]thymidine (NEN, Boston, MA) after harvest (Tomtec, Orange, CT) following an 18-h pulse. Incorporation was measured using a liquid scintillation counter (Wallace, Gaithersburg, MD).

Immunoprecipitations and Western blots

Human purified CD8⁺ T cells or CD8⁺ T cell clones were washed, resuspended in RPMI 1640, incubated on ice for 15 min with either APCs or Ab (as indicated), and warmed to 37°C for the time indicated. The cells were washed twice with cold RPMI containing 1 μM sodium orthovanadate (Sigma, St. Louis, MO) and lysed for 15 min on ice in cold lysis buffer (1% Nonidet P-40, 150 mM NaCl, 25 mM HEPES, pH 7.5, 1 mM EDTA, 1 μM sodium orthovanadate, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). Detergent extracts were clarified by centrifugation at 14,000 × g for 10 min at 4°C. The resulting supernatants were harvested and either used for immunoprecipitations or separated by electrophoresis through 8% SDS-PAGE (Protogel; National Diagnostics, Atlanta, GA). For immunoprecipitation, cellular extracts were incubated with the p70s6k antisera and 25 μl of protein A-agarose for at least 2 h at 4°C, after which the resin was washed three times with lysis buffer. Separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA), immunoblotted with the p70s6k Ab, and detected by ECL (Amersham, Arlington Heights, IL) according to the manufacturer’s instructions.

Results

A subpopulation of Ag-specific CD8⁺human T cell clones proliferates in the presence of high concentrations of rapamycin

A panel of Ag-specific CD8⁺ human T cell clones was single cell cloned from the blood of an HTLV-1-infected HLA-A*0201⁺ individual using an HLA-A*0201/HTLV-1 Tax11–19 tetramer (28). CD8⁺ T cell clones were pretreated with one of two doses of rapamycin or vehicle control, followed by stimulation with PBS- or Tax peptide-pulsed EBV-transformed B cells expressing HLA-A*0201 (KSB line). Antigenic stimulation with either of two doses of Tax peptide induced proliferation of all the clones, and rapamycin at both concentrations inhibited the proliferation of four of the clones (Fig. 1A). Surprisingly, the proliferation of two clones (TP7 and TP60) was resistant to inhibition by very high doses of rapamycin (1 and 10 μM, respectively) (Fig. 1B). Thus, physiological stimulation of human CD8⁺ T cells with cognate Ag presented by APCs could result in proliferation resistant to very high doses of rapamycin.

Rapamycin-resistant proliferation of a human CD8⁺ T cell clone is both restricted to stimulation by cognate MHC and dependent on strength of antigenic stimulation

Rapamycin affects the ability to progress through cell cycle, a progression induced by a combination of signals through the TCR and costimulators, as well as by cytokines secreted in response to stimulation. It was of interest to determine the contribution of the strength of stimulation through the TCR to rapamycin-resistant proliferation. We took advantage of a previous observation that CD8⁺ T cell clones recognizing the HLA-A*0201/Tax11–19 complex can be cross-reactive with the related allogeneic HLA-A*0205 subtype pulsed with Tax11–19 (D. G. Lim, K. Bourcier, G. Buckle, G. Freeman, A. Sette, and D. A. Hafler, manuscript in preparation). The CD8⁺ human T cell clones TP7 and TP60 proliferate in response to HLA-A*0205 pulsed with the Tax11–19 peptide. However, stimulation of the clones with this MHC altered peptide ligand results in quantitatively less thymidine incorporation and is likely to be representative of a weaker signal through the TCR. This model system was used to attenuate the strength of stimulation through the TCR to rapamycin-resistant proliferation. We took advantage of a previous observation that CD8⁺ T cell clones recognizing the HLA-A*0201/Tax11–19 complex can be cross-reactive with the related allogeneic HLA-A*0205 subtype pulsed with Tax11–19 (D. G. Lim, K. Bourcier, G. Buckle, G. Freeman, A. Sette, and D. A. Hafler, manuscript in preparation). The CD8⁺ human T cell clones TP7 and TP60 proliferate in response to HLA-A*0205 pulsed with the Tax11–19 peptide. However, stimulation of the clones with this MHC altered peptide ligand results in quantitatively less thymidine incorporation and is likely to be representative of a weaker signal through the TCR. This model system was used to attenuate the strength of antigenic stimulation to determine whether a weaker signal through the TCR would be susceptible to inhibition by rapamycin. The human CD8⁺ T cell clone TP60 was pretreated with either 10 μM rapamycin or vehicle control, followed by stimulation with Tax11–19-pulsed or nonpulsed EBV-transformed B cells expressing either HLA-A*0201 (KSB line) or HLA-A*0205 (DAH line) (Fig. 2A). Although HLA-A*0201 plus Tax11–19 stimulation resulted in rapamycin-resistant proliferation, HLA-A*0205 plus Tax11–19 resulted in proliferation that was completely abrogated by rapamycin.
In this system, it was also possible to vary the strength of TCR signal by changing the amount of antigenic peptide used to pulse the APCs. We used this approach to confirm that a weaker stimulation through the TCR resulted in a rapamycin-sensitive proliferation. The CD8+ T cell clone TP60 was pretreated with vehicle control, 10 nM, or 1 μM rapamycin, and then exposed to HLA-A*0201 APCs that had been pulsed with the Tax11–19 peptide or PBS control in the presence or absence of rapamycin (as indicated). Proliferation was assessed at 72 h by the incorporation of [3H]thymidine. Average cpm + SE of triplicate wells from a representative experiment are shown.

**FIGURE 1.** Rapamycin-sensitive and rapamycin-resistant proliferation of human CD8+ T cell clones. Six different CD8+ T cell clones were pretreated with rapamycin or ethanol (vehicle control). T cells were cultured with irradiated HLA-A*0201-expressing EBV-transformed B cells (KSB line) pulsed with the Tax11–19 peptide or PBS control in the presence or absence of rapamycin (as indicated). Proliferation was assessed at 72 h by the incorporation of [3H]thymidine. Average cpm + SE of triplicate wells from a representative experiment are shown.

Rapamycin-resistant proliferation of a human CD8+ T cell clone can be modeled by cross-linking CD3

High concentrations of cognate peptide Ag were capable of inducing rapamycin-resistant proliferation of the T cell clones TP60 and TP7, whereas relatively low concentrations of the same peptide Ag or peptide Ag presented by allogeneic MHC induced rapamycin-sensitive proliferation of the same clones. This suggested a strict dependence on specific signaling through the TCR, or a narrow window of antigenic stimulation, required for rapamycin-resistant proliferation. We examined whether resistance to rapamycin could be observed after TCR cross-linking using different concentrations of immobilized anti-CD3 mAb. The human CD8+ T cell clone TP60 was pretreated either with vehicle control, or with 1,
Rapamycin (1 nM) inhibited proliferation by nearly 50%, whereas higher doses of rapamycin completely inhibited proliferation. The major clinical use of rapamycin is currently in the setting of transplantation; therefore, we wanted to test the efficacy of rapamycin at inhibiting a MLR, a surrogate, in vitro test for alloreactivity. CD8⁺ T cells were pretreated with vehicle control (filled symbols) or with 1 μM rapamycin (open symbols), then exposed to one of two different irradiated EBV-transformed B cell lines (DAH and KSB) at increasing concentrations (Fig. 3C). The CD8⁺ T cells responded to both EBV-transformed B cell lines, albeit at a more robust level to KSB. Rapamycin dramatically inhibited proliferation regardless of the allostimulation (open symbols), demonstrating that, as expected, the alloresponse was sensitive to inhibition by rapamycin.

Simultaneously cross-linking CD3 and CD28, but not CD2 or CD8, on human CD8⁺ T cells results in proliferation that is resistant to inhibition by rapamycin

The formation of the immunological synapse impacts upon the outcome of Ag encounter and resulting effector function. Thus, it was of interest to assess the relative contributions of major coreceptors to rapamycin sensitivity/resistance, and determine which, if any, would confer rapamycin resistance. CD28, given its pivotal role in costimulation, was the first cell surface receptor we evaluated. CD8⁺ peripheral blood T cells were pretreated with either vehicle control, or with 1, 10, or 100 nM rapamycin, then stimulated with increasing concentrations of plate-bound anti-CD3 mAb, in the presence of soluble anti-CD28 mAb at the indicated concentrations (Fig. 4A). As expected, the costimulatory signal provided by the addition of 1 or 5 μg/ml soluble anti-CD28 mAb resulted in enhancement of proliferation that was inhibited by rapamycin, with the exception of T cell stimulation by 50 μg/ml anti-CD3 (Fig. 4A). It has been shown that different signals can be generated through CD28, depending on the particular stimulation (CD80, CD86, or different mAbs) (31, 32). In the absence of APCs, to deliver a signal approximating that delivered in vivo, we simultaneously cross-linked CD3 and CD28 with immobilized, as opposed to soluble, Abs (Fig. 4B). This resulted in an increased sensitivity to anti-CD3 mAb stimulation with a shift of the dose response to the left (compare Fig. 3B with Fig. 4B). This was apparent even at 1 μg/ml anti-CD28, slightly enhanced by the use of 5 μg/ml CD28 mAb, and not changed further by 10 μg/ml (data not shown). Now, costimulation with 5 or 10 μg/ml of immobilized anti-CD28 resulted in complete resistance of CD8⁺ T cells to up to 100 nM rapamycin.

Although CD28 engagement provides a major costimulatory signal, several other surface receptors also contribute significantly to T cell responses such as proliferation. Accordingly, several other receptor-ligand pairs have been implicated in the formation of the immunological synapse. For this reason, we examined the ability of other costimulators to stimulate rapamycin-resistant proliferation. CD8⁺ T cells pretreated with vehicle control, 1 nM, 500 nM, or 1 μM rapamycin were incubated with increasing concentrations of anti-CD3 mAb in the presence of either anti-CD2 mAb or anti-CD8 mAb (all immobilized) (Fig. 5). Cocross-linking of CD3 with either CD2 or CD8 augmented proliferation beyond that observed after cross-linking CD3 alone. In both cases, proliferation of the CD8⁺ T cells was inhibited by even 1 nM rapamycin. Thus, CD28 appeared to be unique in its ability to deliver a signal to circulating CD8⁺ T cells for proliferation that is resistant to the inhibitory effects of rapamycin.

FIGURE 2. Rapamycin-resistant proliferation of the CD8⁺ T cell clone TP60 is restricted to stimulation with cognate Ag and dependent on strength of antigenic stimulation. The CD8⁺ T cell clone TP60 was pretreated with rapamycin or ethanol (vehicle control). T cells were cultured with irradiated HLA-A*0201 (KSB line) or HLA-A*0205 (DAH line)-expressing EBV-transformed B cells pulsed with the Tax11–19 peptide or with irradiated HLA-A*0201 (KSB line)- or HLA-A*0205 (DAH line)-treated with rapamycin or ethanol (vehicle control). T cells were cultured using anti-CD3 mAb to stimulate the CD8⁺ T cell clone provided if any, would confer rapamycin resistance. CD28, given its pivotal role in costimulation, was the first cell surface receptor we evaluated. CD8⁺ peripheral blood T cells were pretreated with either vehicle control, or with 1, 10, or 100 nM rapamycin, then stimulated with increasing concentrations of plate-bound anti-CD3 mAb, in the presence of soluble anti-CD28 mAb at the indicated concentrations (Fig. 3A). As expected, the costimulatory signal provided by the addition of 1 or 5 μg/ml soluble anti-CD28 mAb resulted in enhancement of proliferation that was inhibited by rapamycin, with the exception of T cell stimulation by 50 μg/ml anti-CD3 (Fig. 4A). It has been shown that different signals can be generated through CD28, depending on the particular stimulation (CD80, CD86, or different mAbs) (31, 32). In the absence of APCs, to deliver a signal approximating that delivered in vivo, we simultaneously cross-linked CD3 and CD28 with immobilized, as opposed to soluble, Abs (Fig. 4B). This resulted in an increased sensitivity to anti-CD3 mAb stimulation with a shift of the dose response to the left (compare Fig. 3B with Fig. 4B). This was apparent even at 1 μg/ml anti-CD28, slightly enhanced by the use of 5 μg/ml CD28 mAb, and not changed further by 10 μg/ml (data not shown). Now, costimulation with 5 or 10 μg/ml of immobilized anti-CD28 resulted in complete resistance of CD8⁺ T cells to up to 100 nM rapamycin.

10, or 100 nM rapamycin, and then stimulated with increasing concentrations of immobilized anti-CD3 mAb (Fig. 3A). Concentrations of immobilized anti-CD3 as low as 1 ng/ml induced proliferation of the TP60, and at low concentrations of anti-CD3 (≤100 ng/ml) proliferation of the T cell clones was inhibited by rapamycin. However, at higher concentrations of anti-CD3 (≥1 μg/ml), proliferation was resistant to inhibition by 100 nM rapamycin.

The ability to recapitulate the rapamycin-resistant proliferation using anti-CD3 mAb to stimulate the CD8⁺ T cell clone provided a means by which we could extend these studies to CD8⁺ peripheral blood T cells ex vivo. Human peripheral blood CD8⁺ T cells were pretreated with vehicle control, or 1, 10, or 100 nM of rapamycin, followed by stimulation with increasing concentrations of plate-bound anti-CD3 mAb (Fig. 3B). In agreement with published observations describing the relative costimulation independence of CD8⁺ T cells after exposure to strong signals through the TCR (29, 30), immobilized anti-CD3 alone stimulated the proliferation of the CD8⁺ T cells (Fig. 3B). In contrast to our findings with the CD8⁺ T cell clone TP60, rapamycin readily inhibited the CD3-induced proliferation of freshly isolated human CD8⁺ T cells.

The formation of the immunological synapse impacts upon the outcome of Ag encounter and resulting effector function. Thus, it was of interest to assess the relative contributions of major coreceptors to rapamycin sensitivity/resistance, and determine which, if any, would confer rapamycin resistance. CD28, given its pivotal role in costimulation, was the first cell surface receptor we evaluated. CD8⁺ peripheral blood T cells were pretreated with either vehicle control, or with 1, 10, or 100 nM rapamycin, then stimulated with increasing concentrations of plate-bound anti-CD3 mAb, in the presence of soluble anti-CD28 mAb at the indicated concentrations (Fig. 3A). As expected, the costimulatory signal provided by the addition of 1 or 5 μg/ml soluble anti-CD28 mAb resulted in enhancement of proliferation that was inhibited by rapamycin, with the exception of T cell stimulation by 50 μg/ml anti-CD3 (Fig. 4A). It has been shown that different signals can be generated through CD28, depending on the particular stimulation (CD80, CD86, or different mAbs) (31, 32). In the absence of APCs, to deliver a signal approximating that delivered in vivo, we simultaneously cross-linked CD3 and CD28 with immobilized, as opposed to soluble, Abs (Fig. 4B). This resulted in an increased sensitivity to anti-CD3 mAb stimulation with a shift of the dose response to the left (compare Fig. 3B with Fig. 4B). This was apparent even at 1 μg/ml anti-CD28, slightly enhanced by the use of 5 μg/ml CD28 mAb, and not changed further by 10 μg/ml (data not shown). Now, costimulation with 5 or 10 μg/ml of immobilized anti-CD28 resulted in complete resistance of CD8⁺ T cells to up to 100 nM rapamycin.
p70S6 kinase activity does not account for rapamycin-resistant proliferation

Rapamycin inhibits progression through cell cycle. One of the major mechanisms of this action is inhibition of the activation of p70S6k. This kinase phosphorylates the 40S ribosomal subunit S6, permitting translation and promoting cell cycle progression. Rapamycin inhibits the activation of p70S6k, either directly by preventing mTOR activation of p70S6k, or indirectly by interfering with mTOR inactivation of the phosphatase PP2A, which dephosphorylates p70S6k and maintains it in an inactive state. Given its pivotal role in cell cycle control, and that it is a well-accepted target of rapamycin inhibition, we examined phosphorylation of p70S6k.

The phosphorylation of p70S6k results in a mobility shift of the protein on SDS-PAGE that serves as a surrogate for kinase activity. The human CD8\(^+\) T cell clone TP60 was pretreated with either vehicle control or 1 \(\mu\)M rapamycin, and then exposed to PBS- or Tax11–19 peptide-pulsed HLA-A*0201\(^+\) EBV-transformed B cells. Cells were stimulated for 7 h at 37°C, lysed, and separated on 8% SDS-PAGE. Proteins were transferred to PVDF and immunoblotted with anti-p70S6k antisera (Fig. 6A). Tax peptide-pulsed A*0201-bearing B cells stimulated a shift in the mobility of p70S6k, such that more of the detected protein migrated slower on the gel (upper three bands of the four). Importantly, the addition of rapamycin completely inhibited the mobility shift of p70S6k, reducing the p70 S6k band to one form, migrating at the fastest relative speed. This was true despite the fact that under these conditions proliferation was resistant to inhibition by rapamycin.

Biochemical analyses would be facilitated by a system in which there were only T cells in the assay. Fig. 6B shows p70S6k immunoprecipitates from the T cell clone TP60 after stimulation for 24 h with 1 ng/ml or 1 \(\mu\)g/ml plate-bound anti-CD3 alone or cocross-linked with anti-CD8, anti-CD28, or anti-CD2 (5 \(\mu\)g/ml). Proteins were separated by SDS-PAGE, transferred to PVDF, and immunoblotted with anti-p70S6k Ab. Stimulation with immobilized anti-CD3 alone (1 \(\mu\)g/ml) or in combination with any of the other Abs resulted in activation of p70S6k, as reflected by a mobility shift of the kinase. The addition of rapamycin (1 \(\mu\)M) eliminated this mobility shift in every case. Thus, p70S6k phosphorylation was
consistent inhibited by rapamycin, even under conditions in which rapamycin-resistant proliferation occurs, suggesting that rapamycin-resistant proliferation cannot be attributed to residual p70s6k activity. This was further confirmed by performing a similar analysis on immunoprecipitates from CD8\(^+\) T cells pretreated with vehicle control or rapamycin (1 \text{mM}), followed by stimulation with cross-linked CD3 or with cocross-linked CD3 and CD28 for 8 h (Fig. 6C). Again, stimulation results in a mobility shift of p70s6k. This activation is completely inhibited by the addition of 1 \text{mM} rapamycin. Thus, in both fresh CD8\(^+\) T cells and in a human T cell clone, using different antigenic or polyclonal stimulations, we demonstrate that rapamycin-resistant proliferation is not due to residual p70s6k activity, as read out by phosphorylation.

Rapamycin-resistant proliferation of a CD8\(^+\) human T cell clone is supported by IL-2

Our studies showed that the rapamycin-resistant proliferation could not be attributed to residual p70s6k activity. Rapamycin does not interfere with cytokine production, but rather blocks proliferation in response to cytokines. Previous reports found that the up-regulation of the IL-15R\(\alpha\) is resistant to treatment with rapamycin (35), whereas the up-regulation of the IL-2R\(\alpha\) (CD25) is sensitive to inhibition by rapamycin (36), as are pathways triggered in response to either IL-15 or IL-2 (3, 35). To clarify the contribution of IL-15 and IL-2 to the rapamycin-resistant proliferation of the T cell clone, we determined whether blocking either IL-15 or IL-2 could prevent rapamycin-resistant proliferation. Because IL-2 is a major growth factor supporting proliferation of T cells, which is consumed in these cultures to the extent that it is not readily detected at the protein level, our approach was to determine whether neutralizing IL-2 would alter rapamycin sensitivity of the T cell clones. The human CD8\(^+\) T cell clone TP60 was pretreated with 1 \text{nM} or 1 \text{mM} rapamycin, stimulated with the HLA-A*0201\(^+\) EBV-transformed B cell line (KSB), and pulsed either with PBS, or 0.1 or 10 \text{mM} Tax11–19 (Fig. 7). In the cultures, the cells were treated with isotype control, anti-IL-15 mAb, or anti-IL-2 mAb to neutralize IL-15 or IL-2, respectively. As expected, HLA-A*0201 plus Tax 10 \text{mM}, in the presence of isotype control Ab, induced proliferation that was resistant to the effects of even 1 \text{mM} rapamycin. Treatment with anti-IL-15 affected neither basal proliferation nor rapamycin-resistant proliferation. However, the addition of anti-IL-2 both inhibited proliferation in response to the lower concentration of peptide Ag and converted the rapamycin-resistant proliferation into rapamycin sensitive. In the presence of neutralizing anti-IL-2 Ab, even the 1 \text{nM} rapamycin inhibited the 0.1 \text{mM} Tax peptide-specific proliferation completely, and the proliferation triggered by 10 \text{mM} Tax peptide was reduced by 50%. Treatment with 1 \text{mM} rapamycin nearly abrogated all proliferation in the presence of anti-IL-2 Ab. Thus, the growth factor IL-2 was necessary for the Ag-driven rapamycin-resistant proliferation of the T cell clone.

Discussion

Although rapamycin is a potent inhibitor of cellular proliferation, its specific clinical effects suggest that multiple factors determine whether rapamycin blocks T cell activation. In this study, we examined the relative ex vivo contribution of TCR and costimulatory signals to rapamycin’s blockade of T cell activation. We found that
both the strength of signal delivered through the TCR and secondary costimulatory signals determined whether rapamycin either inhibited or, alternatively, enhanced the clonal expansion of CD8<sup>+</sup> T cells. Rapamycin-resistant proliferation was observed despite efficient inhibition of p70<sub>s6k</sub> phosphorylation. Moreover, CD8<sup>+</sup> T cell clonal activation in the context of a blockade of IL-2 signaling converted rapamycin resistance to sensitivity. These data provide a mechanistic framework to better explain the clinical effects of rapamycin in preventing transplant rejection.

CD8<sup>+</sup> T cells are involved in mediating graft infiltration and rejection. Thus, it was of importance to determine under which conditions rapamycin inhibited clonal expansion of CD8<sup>+</sup> T cells. The proliferation of a panel of CD8<sup>+</sup> T cell clones recognizing the Tax11–19 peptide in the context of HLA-A*0201 was examined. Approximately one-fourth of the CD8<sup>+</sup> clones were resistant to rapamycin inhibition after activation via Ag/MHC engagement, and the same clones were uninhibited by rapamycin after anti-CD3-mediated TCR cross-linking, even at high concentrations of drug. Titrating the amount of peptide presented to the T cell clone in the context of cognate MHC or lower degrees of TCR cross-linking resulted in rapamycin sensitivity. We assessed whether changes in the strength of signal delivered through the TCR by allogeneic presentation of Ag would convert rapamycin resistance to sensitivity using the allogeneic HLA-A*0205 MHC to present the Tax11–19 peptide to HLA-A*0201-restricted T cell clones. This resulted in conversion of the rapamycin-resistant signal observed with cognate ligand to a rapamycin-sensitive signal. Taken together, these data suggest a partial dependence on strength of TCR signal for rapamycin sensitivity.

We consistently observed that rapamycin enhanced proliferation, albeit to a minor degree, in CD8<sup>+</sup> T cell clones stimulated with either peptide/MHC complexes or anti-CD3 mAb. This suggests that the doses of rapamycin used, although much higher than necessary for inhibition of proliferative responses, were not toxic to the cells. In the experiments using peptide/MHC to stimulate the T cell clones, the APC were loaded with peptide, then washed, suggesting that rapamycin was not blocking fratricide that occurs with T-T cell presentation of Ag. However, it has been reported that the type II TNF-R and TNF-related apoptosis-inducing ligand induction are inhibited by rapamycin in murine T cells (37, 38). If this extends to human T cells, the failure to induce TNF-R and/or TNF-related apoptosis-inducing ligand in the presence of rapamycin may result in the indirect inhibition of activation-induced cell death in our system, and this is currently under investigation.

It was possible that the rapamycin resistance related to changes in T cell function associated with long-term in vitro growth of T cell clones. This was an unlikely explanation for our results, given that the same T cell clone was able to respond with either rapamycin-resistant or rapamycin-sensitive proliferation, depending on the stimulation conditions. Nevertheless, it was of importance to examine circulating CD8<sup>+</sup> T cells ex vivo, stimulated with either TCR cross-linking or with allogeneic stimulation, which enabled us to further confirm the ability of CD8<sup>+</sup> T cells to proliferate in a rapamycin-resistant manner. CD8<sup>+</sup> T cell clonal expansion ex vivo, as measured by [3H]thymidine incorporation after cocross-linked anti-CD3 (10 or 50 μg/ml) and anti-CD28 (1 μg/ml), was partially blocked by rapamycin, confirming that only a subset of circulating T cells is inhibited by rapamycin. Further efforts to sort and characterize rapamycin-resistant CD8<sup>+</sup> T cells are in progress.

The in vivo T cell/APC immunologic synapse consists of both Ag-specific receptor-MHC/peptide complexes and receptor-ligand pairs formed by costimulatory molecules (including CD28, CD2, and CD8) that influence the nature of the T cell signal. We directly investigated whether CD28 engagement could reverse rapamycin sensitivity of TCR cross-linking ex vivo of CD8<sup>+</sup> T cells. Both soluble and immobilized anti-CD28 were evaluated in recognition of their different potential to contribute to T cell activation in the absence of APCs. Although CD8<sup>+</sup> T cell proliferation induced by triggering the TCR with immobilized anti-CD3 mAb was highly sensitive to rapamycin inhibition, initiating CD28 signals with immobilized anti-CD28 induced rapamycin resistance. Anti-CD8 or anti-CD2 coengagement did not result in rapamycin resistance of CD8<sup>+</sup> T cells ex vivo. In total, these experiments indicate that changing either the strength of signal delivered through the TCR or engagement of other T cell surface receptors, such as CD28, can confer rapamycin-resistant proliferation. We focused on only a small number of well-characterized components involved in generating signals through the immune synapse, and predict that engagement of other, unexamined costimulatory molecules may similarly contribute to rapamycin-resistant proliferation.

Rapamycin-resistant T cell proliferation did not correlate with p70<sub>s6k</sub> phosphorylation despite its critical role in cell cycle transition. That is, rapamycin-resistant proliferation was observed under conditions resulting in efficient inhibition of p70<sub>s6k</sub> phosphorylation. Because phosphorylation on multiple serine and threonine residues is required for p70<sub>s6k</sub> enzymatic activity, this confirms that p70<sub>s6k</sub> was not activated in the presence of rapamycin. This
demonstrates that p70s6k activation can be uncoupled from proliferation in both freshly isolated CD8\(^+\) T cells and CD8\(^+\) T cell clones. Although this uncoupling has been shown in other malignant cell types such as in erythroleukemic cell lines (39), as well as in activated T cells already cycling (40), this is, to our knowledge, the first demonstration of rapamycin resistance in resting, nontransformed T cells. It remains possible that other members of the S6 kinase family with redundant function compensate for p70 inactivity; however, these family members are also sensitive to rapamycin and therefore unlikely to account for the escape from the effects of rapamycin in our system. The inhibition of p70s6k phosphorylation argues that rapamycin, as predicted, is inhibiting mTOR and that other pathways parallel to but not downstream of p70s6k are capable of promoting cell cycle transition and proliferation.

Blocking the T cell growth factor IL-2, but not IL-15, converted the rapamycin-resistant proliferation of CD8\(^+\) T cell clones to rapamycin sensitive. These data show that the Ag-driven rapamycin-resistant proliferation requires the presence of IL-2, p70s6k activity is downstream of the IL-2R, yet we observed inhibition of p70s6k activity in the presence of rapamycin. These data suggest that pathways parallel to mTOR/p70s6k that are activated downstream of signaling through the IL-2R account for the ability of a subpopulation of CD8\(^+\) T cells to escape cell cycle arrest by rapamycin. A candidate protein in this pathway is c-myc, a regulator of proliferation downstream of IL-2R signaling. c-myc expression has been shown to correlate with rapamycin resistance in human tumor lines (childhood rhabdomyosarcoma and neuroblastoma) (41). Further studies are underway to assess the involvement of c-myc in the rapamycin-resistant proliferation of CD8\(^+\) human T cells described in this work.

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Immunosuppressed patients are often highly susceptible to both dangerous microbial infections and carcinogenic events, as the entire immune response is attenuated without specificity. Our studies suggest that rapamycin inhibition of CD8+ T cell responses can be reversed with a strong danger signal with engagement of the TCR alone or in combination with a strong costimulatory signal. This may explain why secondary infections have not been a common occurrence with rapamycin, as it provides a mechanism for the CD8+ arm of the immune system to combat viral infection despite systemic immunosuppression (reviewed in Ref. 42).

Although in this report we studied CD8+ T cells, the effect of rapamycin on CD4+ T cells is of obvious importance. In preliminary investigations, we observed that CD4+ human peripheral blood T cells, as well as a panel of CD4+ T cell clones, appear to be more readily inhibited by rapamycin, even under conditions that elicit rapamycin-resistant proliferation from CD8+ T cells (data not shown). Further investigations are in progress to formally compare the responses of CD8+ and CD4+ T cell populations.

The rapamycin-resistant proliferation of CD8+ T cells in these studies was observed with concentrations of the drug that approximate or exceed typical circulating levels in patients treated therapeutically with the immunosuppressant. This may be of clinical importance with respect to determination of application-specific dosages. Our results suggest that further studies on rapamycin and its actions at the cellular level are necessary to fully understand how the drug is capable of acting as a powerful immunosuppressant under some conditions, yet allows some immune responses to escape inhibition. This is particularly important as, more recently, rapamycin is under consideration as a potential therapeutic agent for treatment of autoimmune diseases such as type 1 diabetes and multiple sclerosis. Knowledge of the details of the specific mechanisms of action of rapamycin may facilitate tailoring of combination therapies for diseases with attention to variations among applications. Furthermore, reliable information about what determines rapamycin resistance vs susceptibility may help predict the failure or success of rapamycin treatment in a particular individual.

Acknowledgments

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