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Apoptosis of CD4⁺CD25^{high} T cells in response to Sirolimus requires activation of T Cell Receptor and is modulated by IL-2

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Abstract

Targeted molecular therapies inhibit cancer cell proliferation and survival but may interfere with proliferation and survival of lymphoid cells. Treatment with Sirolimus or Sorafenib was cytotoxic to CD4⁺CD25^{high} T cells, and was growth inhibitory for CD4⁺ and CD8⁺ T cells. The cytotoxicity depended on CD3/CD28 stimulation and was detectable as early as 12 hours post-treatment, with 80–90% of the CD4⁺CD25^{high} T cells killed by 72 hours. Cell death was due to apoptosis, based on time-dependent increase in Annexin V and 7AAD staining. Addition of IL-2 prevented the apoptotic response to Sirolimus, potentially accounting for reports that Sirolimus stimulates T cell proliferation. These results predict that Sirolimus or Sorafenib reduce the CD4⁺CD25^{high} T cells if administered prior to antigenic stimulation in an immunotherapy protocol. However, administration of IL-2 protects these CD4⁺CD25^{high} T cells from the cytotoxic effects of Sirolimus, a response that needs to be taken into consideration in therapeutic protocols.

Keywords

Sorafenib; Apoptosis; CD4⁺; CD8⁺; CD3/CD28; Annexin V; 7AAD

Introduction

Novel targeted therapies for cancer include immune therapy and small molecule targeted therapies. One approach to immunization therapy is to use dendritic cells or peptide immunogens to stimulate an immune response against defined cancer antigens. An alternate approach is adoptive T cell therapy, which involves autologous or heterologous transplantation of primed cells. Targeted therapies inhibit cell signaling pathways critical for cancer cell growth, division, and metastasis. There is rationale for considering combinations of these approaches. Inhibitors of cell signaling pathways for growth, proliferation, or survival might enhance immunotherapy by making tumors more susceptible to killing by cytotoxic CD8⁺ T cells. However, inhibitors of cell signaling might interfere with the immune response because many of the pathways critical for tumor cell growth and division are also critical to T cell activation and expansion.

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We performed studies to assess the potential negative effects of Sirolimus and Sorafenib, two signal transduction inhibitors currently in clinical practice and development, on T cell proliferation. Sirolimus (Rapamycin) is an FDA-approved agent, used since the late 1990s as immunosuppressive therapy to prevent rejection of transplanted organ allografts [4,15]. Another inhibitor of mTOR signaling, temsirolimus (CCI-779, Wyeth Pharmaceuticals), is a derivative of Sirolimus and is being evaluated in clinical trials as a cancer therapeutic. Sirolimus binds to a cytoplasmic receptor, FKBP12, and the drug-protein complex inhibits the function of mTOR kinase, an important cellular regulator of translation and transcription [3]. Sorafenib (BAY43-9006) was approved by the FDA in December 2005 for treatment of renal cell cancer [7] and in November 2007 for unresectable hepatocellular carcinoma. It was developed as a B-Raf kinase inhibitor in part because B-Raf has been identified as mutated and over-expressed in multiple cancers, especially melanoma. Sorafenib also inhibits VEGF-R kinases (1/2/3) and other tyrosine kinases, all of which may contribute to its therapeutic actions [1,25]. The effects of Sorafenib on lymphocyte subsets have not been explored.

The T cell population of CD4⁺CD25^{high} has attracted particular attention because of their regulatory activity as tumor suppressor cells [8,12]. One desirable scenario would be to selectively inhibit this population of cells without eliminating the CD4⁺ or CD8⁺ T cells to improve the efficacy of immunization therapy. One would expect that an mTOR inhibitor such as Sirolimus would suppress the proliferation of T cells, however Sirolimus has been reported to selectively expand the CD4⁺CD25^{high} T cells in culture [2,21]. Here we examine the effects of Sirolimus and Sorafenib on the proliferation and survival of CD4⁺CD25^{high} T cells compared to CD4⁺ and CD8⁺ T cells.

Materials and Methods

Cell Separation

Peripheral blood mononuclear cells (PBMC) were isolated from healthy human volunteers, using the following sources: Ficoll gradient centrifugation of whole blood, anonymous donor buffy coats from Virginia Blood Services (Richmond, VA), or leukapheresis of volunteers, either locally or commercially from BRT Laboratories (Baltimore, MD). These were collected under IRB approved protocols (HIC 10598, 12382).

T cell subsets were isolated from PBMC by the Miltenyi Biotech MACS system according to the manufacturer's instructions (Auburn, CA, catalog # 130-091-154 for CD8⁺ T cells; Catalog # 130-091-155 and 130-090-445 for CD4⁺CD25⁻ T cells, and catalog # 130-091-301 for CD4⁺CD25^{high} T cells). The resulting T cell subpopulations were assessed for purity by flow cytometry and were only used if $\geq 90\%$ pure. All of the cells were cultured in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Gemini Bio-Products, Woodland, CA), 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 μ g/ml) at 37 °C in 5% CO₂, unless otherwise indicated.

Flow Cytometry

Cell surface staining was performed according to established protocols. 2×10^5 cells per condition were incubated for 30 min at 4°C with different combinations of the following antibodies in the dark (CD3-APC (BD Biosciences # 555342), CD4-PE-CY7 (Caltag #MHCD0412), CD4-FITC (BD Biosciences # 555346), CD4-PE-CY5 (Beckman Coulter # IM2636), CD8-FITC (Beckman Coulter #TO20222), CD8-APC (BD Biosciences # 555369), and CD25-PE (Miltenyi # 120-001-311)). Following two washings with PBS containing 0.1% BSA and 0.1% Sodium Azide (FACS Buffer) cells were resuspended in 2% paraformaldehyde/PBS (Sigma-Aldrich), and analyzed with the FACS Calibur (Flow Cytometry Core Facility) and FlowJo software. Intracellular human Foxp3 staining was performed: lymphocytes were

stained for 30 minutes with CD25-PE (Miltenyi) and CD4-Fitc (BDPharmingen), and washed in FACS buffer (PBS + 0.1% BSA + 0.1% sodium azide). Surface-stained cells were fixed for 30 minutes using the FoxP3 fixation/permeabilization kit from eBioscience (Cat. # 00-5521) according to their instructions. Fixative was removed by washing two times in permeabilization (Perm) buffer. Cells were incubated in Perm buffer containing 4% normal rat serum for 10 minutes at 4°C, followed by the addition of an equal volume of 1:5 – 1:10 dilution (in Perm buffer) of Foxp3-APC (eBioscience catalog 88-8999-40; PCH101) antibody. After 30 minutes at 4°C, cells were washed twice in Perm buffer, then once in FACS buffer. Cells were acquired on a FACS Calibur flow cytometer and data processed by FloJo software.

Reagents and Inhibitors

Sorafenib (BAY43-9006) was purchased from Calbiochem (Catalog # 553011). Sirolimus (rapamycin) was purchased from LC Laboratories (Woburn, MA; R-5000). Stock solutions were made of each agent in DMSO. For all assays, the negative control was a solution containing DMSO at the same concentration (“vehicle”). IL-2 was purchased from Chiron (Emeryville, CA; Proleukin) and used at 1000 IU/ml.

Cell Proliferation Assays

For experiments to examine the effects of the signal transduction inhibitors on lymphocyte cell proliferation, lymphocytes were plated (30,000 cells per well) in triplicate in flat-bottom 96-well plates (Costar 3610); in RPMI1640 (Sigma), medium supplemented with 10% FBS, glutamine, and pen-strep; they were treated with inhibitor or vehicle at the indicated concentrations then, after an hour, were either stimulated or not with CD3/CD28 Dynabeads (Invitrogen catalog #111-31D). Exogenous cytokines were not used. Lymphocytes were cultured for 72 hours and then cell numbers were assayed by measure of intracellular ATP using Cell Titer Glo (Promega Catalog# G7571; Madison, WI), according to the instructions provided by the manufacturer. The triplicate values were all within 8.5%, and the mean values were calculated and plotted with error bars representing the standard deviation of triplicate samples from at least 3 independent experiments. For time-course studies, all lymphocytes were plated and treated at the same time, and plates were read by the luminometer at the time points indicated (6, 12, 24, 36, 48hrs later, etc). The time-course experiments were done two times, with triplicate wells for each time point, for each experiment.

Apoptosis Assays

T cells were treated with vehicle, 10 nM Sirolimus or 10 nM Sorafenib and after an hour stimulated with CD3/CD28 beads and incubated for 12 or 24 hours as indicated. Cells were then surface stained for CD4 (CD4-FITC), CD25 (CD25-PE), and CD8 (CD8-APC-Cy7) as described above. After surface staining cells, they were washed once in FACS buffer (0.1% BSA and 0.1% NaN₃ (azide) in PBS), once in PBS, and then once more in freshly prepared binding buffer, (10x solution from BD Biosciences Cat. #51-66121E). Annexin V-APC (BD Biosciences Cat.# 550474) was added at a final dilution of 1:10 or 1:20 from the original stock in 1X binding buffer containing 7AAD (1 ug/ml; CalBiochem catalog #129935). The dilution of Annexin V-APC used was based on the number of cells. Cells were pelleted, the buffer was decanted, and cells were gently suspended in residual buffer to which was added 0.02 ml of Annexin V-APC and 7AAD. Appropriate compensation controls and FMOs were performed in conjunction.

Human Subjects

All of the research involving human subjects was approved by the University of Virginia's IRB (Human Investigation Committee) in accordance with assurances filed with and approved by the Department of Health and Human Services.

Results

Response of CD4⁺ CD25^{high} T cells to Sirolimus or Sorafenib

We separated peripheral blood mononuclear cells (PBMCs) from healthy donors and isolated CD4⁺CD25^{high} T cells as described previously using the Miltenyi MACS system [5,6,8,9,11, 16-18,26,27]. Cells were analyzed by flow cytometry and found to be $\geq 90\%$ CD4⁺CD25^{high} (Figure 1a). Purified CD4⁺CD25^{high} T cells did not proliferate without stimulation, but when stimulated with CD3/CD28, the number of cells increased up to 3 fold after 72 hours (responses were variable among different donors). Treatment with Sirolimus or Sorafenib for one hour prior to stimulation by CD3/CD28 caused a similar dose-dependent reduction in the number of these T cells (Figure 1b). The dose response curve revealed both inhibition of proliferation and cytotoxicity: below 3 nM Sirolimus or Sorafenib, there was inhibition of proliferation, at 3 nM there was essentially no proliferation (Figure 1b dashed line) and at higher concentrations of 10 nM and 100 nM, there was a near complete net loss in the number of viable CD4⁺CD25^{high} T cells. Thus, doses of Sirolimus and Sorafenib below 10 nM were cytotoxic for CD3/CD28-stimulated CD4⁺CD25^{high} T cells. The time dependence of the cytotoxic response was examined (Figure 1c) and showed that there was no net loss of CD4⁺CD25^{high} T cells prior to 12 hours and a steep decline in the number of cells from 12 to 72 hours, at which time only about 20% of the cells survived. The response was very similar for 10nM Sirolimus and for 10 nM Sorafenib.

CD4⁺ and CD8⁺ human lymphocytes proliferate in the presence of Sirolimus or Sorafenib

CD4⁺ and CD8⁺ T cell subsets were isolated from peripheral blood mononuclear cells (PBMCs) from healthy donors. Isolation of CD4⁺ T cells excluded CD25⁺ cells, and hereafter these are referred to as CD4⁺ T cells. Representative flow cytometry data demonstrate purity $> 90\%$ for CD4⁺ CD8⁻ cells (Figure 2a) and for CD4⁻CD8⁺ cells (Figure 2b). These T cell subsets were pre-treated with Sirolimus or Sorafenib, then stimulated with CD3/CD28 to engage the T cell receptor. CD4⁺ T cells increased in number by 150–200% after T cell receptor engagement, as shown in Figure 2c (open bar). Pre-treatment with either Sirolimus (hatched bars) or Sorafenib (solid bars) at 0.01 nM to 100 nM caused a dose-dependent decrease in proliferation (Figure 2c). At these very low doses, a 10^4 fold change in concentration resulted in only about a 50% decrease in proliferation. We note that therapeutic doses of these agents produce serum levels (100 uM Sirolimus [19], 10 uM Sorafenib [10,22]) that are 100–1000 times higher than the concentrations tested here. When CD4⁺ T cells were treated with Sirolimus plus Sorafenib together at any of the concentrations in this range, there was a net decrease in cell number relative to the initial input (data not shown). CD8⁺ T cells were more responsive to CD3/CD28 stimulation, increasing in number nearly 500% (Figure 2d, open bar). Addition of even the lowest dose (0.01 nM) of Sirolimus (hatched bars) or Sorafenib (solid bars) decreased this response by nearly 50% (Figure 2d). A 10^4 increase in dose resulted in little further inhibition. Thus, both CD4⁺ and CD8⁺ T cells survived in the presence of these doses of Sirolimus or Sorafenib, and CD8⁺ T cells continued to proliferate under these conditions.

The time dependence of the inhibitory effect of Sirolimus and Sorafenib on CD4⁺ and CD8⁺ T cell proliferation was examined. CD4⁺ T cells treated with vehicle, as a control, and stimulated with CD3/CD28 began to increase in number after 12 hours (Figure 2e, dashed line). In the presence of either 10 nM Sirolimus (open circles) or 10 nM Sorafenib (closed squares), the number of CD4⁺ T cells did not change significantly over 72 hours (Figure 2e). By comparison, CD8⁺ T cells resisted inhibitory effects of low dose Sirolimus or Sorafenib and increased in cell number parallel to the control starting 4 hours after addition of CD3/CD28 beads (Figure 2f). These data demonstrate that Sirolimus and Sorafenib were not cytotoxic for

either the CD4⁺ or CD8⁺ T cell populations, a distinctly different response than with the CD4⁺CD25^{high} T cells (Figure 1c).

Apoptosis of CD4⁺CD25^{high} T cells in response to Sirolimus or Sorafenib

The time-dependent depletion of CD4⁺CD25^{high} T cells in response to either Sirolimus or Sorafenib was due to apoptosis. Isolated subsets of CD4⁺, CD8⁺, and CD4⁺CD25^{high} T cells were treated with Sirolimus or Sorafenib and after an hour stimulated with CD3/CD28. After 12 or 24 hours, apoptotic cells were stained with Annexin V and non-viable cells with 7AAD (Figure 3). Compared to treatment with vehicle (left column), CD4⁺ T cells treated with either Sirolimus (center column) or Sorafenib (right column) exhibited an average (n=3) 4 to 5 fold increase in the percentage of apoptotic and non-viable cells (Figure 3a). Thus, the lack of change in the number of CD4⁺ T cells cultured in the presence of Sirolimus or Sorafenib (see Figure 2e) reflects a balance of cell death and cell proliferation. Treatment of CD8⁺ T cells with either Sirolimus or Sorafenib caused an average (n=3) 2–3 fold increase in apoptotic and non-viable cells (Figure 3b). The low observed incidence of apoptosis in the CD8⁺ population is consistent with a net increase in cell number in the presence of Sirolimus or Sorafenib (Figure 2f, see above). CD4⁺CD25^{high} T cells treated with either Sirolimus or Sorafenib had an average (n=2) 4–6 fold increase in apoptotic and non-viable cells at 12 hours (Figure 3c). At 24 hours after treatment with Sirolimus or Sorafenib, the percentage of apoptotic and non-viable cells increased further to 36% and 27% respectively (Figure 3d). Thus, Sirolimus and Sorafenib induced apoptosis and cell death in CD4⁺CD25^{high} T cell population.

Apoptosis of CD4⁺CD25^{high} T cells is dependent upon CD3/CD28 stimulation

We treated isolated CD4⁺CD25^{high} T cells from healthy donors with Sirolimus or Sorafenib and after an hour, stimulated them (or not) with CD3/CD28. Without stimulation by CD3/CD28, the number of apoptotic or non-viable CD4⁺CD25^{high} T cells at 24 hours was not increased by treatment with either Sirolimus (center column) or Sorafenib (right column) compared to vehicle (left column) (Figure 4a). CD4⁺CD25^{high} T cells from the same donor stimulated with CD3/CD28 showed a significant increase in the number of apoptotic and non-viable cells after treatment with Sirolimus or Sorafenib (Figure 4b). Similar results were obtained with CD4⁺CD25^{high} T cells from two other normal donors (data not shown). Thus, CD4⁺CD25^{high} T cells did not undergo apoptosis in response to Sirolimus or Sorafenib unless their T cell receptors were activated by CD3/CD28 co-stimulation.

IL-2 abrogates the apoptotic response of CD4⁺CD25^{high} T cells to Sirolimus

IL-2 is sometimes added to stimulate primary cultures of T cells. To test the effect of IL-2 on the cytotoxicity of Sirolimus, CD4⁺CD25^{high} T cells were stimulated with CD3/CD28 with and without IL-2 (Figure 5). Sirolimus produced dose-dependent inhibition of CD4⁺CD25^{high} T cell proliferation in response to TCR activation (Figure 5, columns 3–5), and this response was completely blocked by addition of IL-2 to the cultures (Figure 5, columns 6–8). In controls without TCR activation, apoptosis of CD4⁺CD25^{high} T cells was not increased by Sirolimus, with or without IL-2 (not shown). Thus, IL-2 prevented the cytotoxic effect of Sirolimus on CD3/CD28-stimulated CD4⁺CD25^{high} T cells.

Discussion

We began these *in vitro* studies because of our interest in using targeted therapies in conjunction with immunotherapy for melanoma treatment. One of our concerns was that targeted agents would have immunosuppressive effects and thereby might diminish responses to immunotherapy. Here, our goal was to evaluate Sirolimus and Sorafenib for their effects on different populations of T cells from peripheral blood. Sirolimus and Sorafenib are both FDA approved agents and are used clinically at doses that produce serum concentrations in the range

of 100 micromolar. At doses more than 1000 times lower, we found *in vitro* cytotoxicity of primary CD4⁺CD25^{high} T cells, but not CD4⁺ or CD8⁺ T cells. We stimulated proliferation of these different T cell subsets using an artificial stimulus of CD3/CD28 dynabeads as an experimental model to mimic the *in vivo* activation of the T cell receptor. This method provided us with a uniform stimulus that increased cell number for each of the subsets of T cells from peripheral blood. The protocol elicited proliferation of the CD4⁺CD25^{high} T cell population [8,12,13]. This population of cells was consistently 60–80% Foxp3⁺ by antibody staining in flow cytometry (data not shown). Expression of Foxp3 has been used as one marker to distinguish regulatory T cells from activated CD4⁺CD25⁺ T cells. Dependence on Foxp3 as a marker for human regulatory T cells has been challenged [14,20,24,28]. It is possible that our isolated CD4⁺CD25^{high}Foxp3⁺ T cells function *in vivo* as regulatory/suppressor cells, but it is not possible to know whether or not there are other cells that provide the same function. Regardless, stimulation of the TCR by CD3/CD28 prompted apoptosis of the CD4⁺CD25^{high} T cells when they were treated with Sirolimus or Sorafenib at < 10 nM. This apoptotic effect was completely ablated by the addition of IL-2 to the culture medium. Thus, we propose that the inclusion of IL-2 in the culture conditions accounts for the unusual stimulatory response to Sirolimus. This effect could be due to activation of proliferation signals or a change in the balance of pro- versus anti-apoptotic signals. We imagine that there is opposing signaling by the T cell receptor and the IL-2 receptor that determines the apoptotic response to Sirolimus (Figure 6).

The mechanism for differential susceptibility of T cell subsets to Sirolimus and Sorafenib remains unknown. There was a dramatic loss of CD4⁺CD25^{high} T cells above a 3 nM threshold dose. This response suggests titration of a limiting factor essential for CD4⁺CD25^{high} T cell survival that is induced by stimulation with CD3/CD28. Thus, an intriguing question is whether there is a common target for Sirolimus and Sorafenib. One simple explanation would be a protein inhibited by both drugs. Sirolimus and Sorafenib both inhibit protein kinases. These chemical agents have distinctive structural backbones and act by different mechanisms. Sirolimus is a macrolide that targets a prolyl-isomerase (FKBP12) that, in turn, binds and inhibits mTOR kinase at a site separate from the kinase domain. On the other hand, Sorafenib (aka: BAY43-9006, N-(3-trifluoromethyl-4-chlorophenyl)-N'-(4-(2-methylcarbamoyl pyridin-4-yl)oxyphenyl)urea), inhibits Raf and VEGFR kinases by competing with ATP for binding at the active site in the kinase domain. These considerations make it unlikely that Sirolimus and Sorafenib compete for a single intracellular receptor to trigger apoptosis in CD4⁺CD25^{high} T cells. It is possible that there is an unrecognized common target for Sirolimus and Sorafenib, but our results predict that: 1) binds both compounds with nanomolar affinity, 2) be induced by CD3/CD28 stimulation and 3) be essential for CD4⁺CD25^{high} T cell survival, and not survival of CD4⁺ or CD8⁺ T cells. Comparative proteomic analyses of different T cell subsets could be employed to identify new nanomolar affinity targets for Sirolimus and Sorafenib. Alternatively, inhibition of the recognized targets of Sirolimus and Sorafenib (FKBP12/mTOR and Raf) might, by separate pathways, extinguish an unidentified signal that is critical for survival of CD4⁺CD25^{high} T cells.

Sirolimus, although it is a well-known immunosuppressant drug that *in vivo* prevents proliferation of T cells, has been reported to selectively expand CD4⁺CD25^{high} T cell populations in culture [2,21]. These studies have reported stimulatory effects of Sirolimus on T cells. Naïve CD4⁺ T cells from DO11.10 TCR transgenic mice were treated with antigen presenting cells plus ovalbumin (OVA) peptide in the presence or absence of 100 nM Sirolimus for three weeks, followed by an additional week with added IL-2. At the end of the protocol, the same total number of T cells was recovered, indicating that Sirolimus did not inhibit proliferation [2]. In fact, the number of CD4⁺CD25^{high} T cells was greater in the presence of Sirolimus relative to the control [2], suggesting that the CD4⁺CD25^{high} T cells were selectively stimulated by Sirolimus or that Sirolimus promoted conversion of the CD4⁺ cells to

CD4⁺CD25^{high}. The latter hypothesis was supported by another study that reported Sirolimus induced expression of regulatory markers on helper CD4⁺ T cells [23]. Furthermore, human CD4⁺CD25⁺Foxp3⁺ regulatory T cells in long-term culture with IL-2 proliferated in the presence of 1 nM Sirolimus [21], but were inhibited by 10 nM Sirolimus. This result is consistent with our observation of a sharp transition between proliferation and apoptosis over a narrow dose range of Sirolimus.

These and other more recent reports seem to contradict our observations of inhibitory and cytotoxic responses of CD4⁺CD25^{high} T cells to nanomolar concentrations of Sirolimus. However, in those reports, IL-2 was included in long-term cultures of T cells prior to their challenge with Sirolimus [2,21]. We therefore tested IL-2 in our cultures, and found it made the CD4⁺CD25^{high} T cells resistant to cytotoxic effects of Sirolimus. Important differences between the two types of experiments still remain. There is the possibility that long term cultures (weeks) with IL-2 produce major changes in gene expression that account for the subsequent response to Sirolimus. In our experiments, Sirolimus and IL-2 were added within an hour of each other, making it less likely that activation of transcription and translation by IL-2 was responsible for the resistance to Sirolimus. We think it more likely that signaling from the activated IL-2 receptor modified existing proteins to make CD4⁺CD25^{high} T cells resistant to the inhibition by Sirolimus. We propose that IL-2 stimulates anti-apoptotic signals which make the CD4⁺CD25^{high} T cells resistant to the pro-apoptotic effects of Sirolimus (Figure 6).

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Figure 1. Threshold effect of Sirolimus or Sorafenib on CD3/CD28 induced proliferation of CD4⁺CD25^{high} T cells

a, CD4⁺CD25^{high} T cells were isolated and stained with CD4-FITC (X-axis) and CD25-PE (Y-axis) and analyzed by flow cytometry. **b**, CD4⁺CD25^{high} T cells were treated with Sirolimus or Sorafenib prior to TCR engagement with CD3/CD28 beads. Cell number ($\times 10^4$) is plotted on the y-axis. The concentrations of Sirolimus (dotted line, open diamonds) or Sorafenib (solid line, closed squares) are plotted in log scale on the x-axis from 0.01 to 100 nM. Data are averages $\pm$ standard deviations of triplicate samples from independent experiments (n=2 for 3 nM, n=3 for others). **c**, Isolated CD4⁺CD25^{high} T cells were treated with 10 nM Sirolimus (open circles) or 10 nM Sorafenib (closed squares), compared to No Drug (vehicle, dashed

line) and then evaluated for cell number over time in hours. Data are averages of triplicate samples from two independent experiments.

Figure 2. Inhibition of CD4⁺ and CD8⁺ T cell proliferation by Sirolimus or Sorafenib

a, CD4⁺ T cells were separated as described in the methods and analyzed by flow cytometry after staining with CD4-FITC (Y-axis) and CD8-APC (X-axis). The percentage of cells in each quadrant is designated. **b**, CD8⁺ T cells were separated as described in the methods and analyzed by flow cytometry after staining with CD4-Pe-Cy5 (Y-axis) and CD8-FITC (X-axis). **c**, CD4⁺ T cells were evaluated for increase in cell number after treatment with vehicle (No Drug, white bar), Sirolimus (hatched bars), or Sorafenib (black bars), with % change in cell number plotted at different concentrations of agent (nM). **d**, CD8⁺ T cells were evaluated for increase in cell number after treatment with vehicle (No Drug, white bar), Sirolimus (hatched bars), or Sorafenib (black bars) with % change in cell number plotted at different concentrations

(nM). Data are calculated as the mean $\pm$ the standard deviation of triplicate values from three independent experiments. Isolated CD4⁺ T cells (**e**) and CD8⁺ T cells (**f**) were treated with 10 nM Sirolimus (open circles) or 10 nM Sorafenib (closed squares), compared to No Drug (vehicle, dashed line) and then evaluated for cell number over time in hours. Data are averages of triplicate samples from two independent experiments.

Figure 3. Treatment with Sirolimus or Sorafenib results in selective increases in Apoptosis of CD4⁺CD25^{high} T cells

CD4⁺T cells (**a**, n=3), CD8⁺ T cells (**b**, n=3), and CD4⁺CD25^{high} T cells (**c**, **d** (n=2 each)) are stained with Annexin V-APC (X-axis) and 7AAD (Y-axis) 12 hours (**a**, **b**, and **c**) or 24 hours (**d**) after treatment with vehicle (left column), 10 nM Sirolimus (center column), or 10 nM Sorafenib (right column). Cells were stimulated with CD3/CD28 one hour after treatment with vehicle or drug.

Figure 4. Selective Apoptosis of $CD4^+CD25^{high}$ T cells is dependent upon stimulation by CD3/CD28
a, $CD4^+CD25^{high}$ T cells were isolated from a normal donor and stained with Annexin V-APC (X-axis) and 7AAD (Y-axis) 24 hours after treatment with vehicle (left column), 10 nM Sirolimus (center column), or 10 nM Sorafenib (right column). **b**, $CD4^+CD25^{high}$ T cells from the same donor were treated as described in **a** except 1 hour after addition of drugs, cells were stimulated with CD3/CD28 and analyzed at 24 hours. These results were replicated with $CD4^+CD25^{high}$ T cells isolated from 2 additional normal donors.

Figure 5. Protective effects of IL-2 on CD4⁺CD25^{high} T cells treated with Sirolimus
 CD4⁺CD25^{high} T cells were treated with Sirolimus (0, 1 or 10 nM), CD3/CD28 beads (columns 2–8), and 1000 IU IL-2/ml (columns 6–8) as indicated in the figure. Data are averages of total cell number $\pm$ standard deviations of triplicate samples from three independent experiments.

Figure 6. Model for effects of TCR activation and IL-2 on responses of CD4⁺CD25^{high} T cells to Sirolimus and Sorafenib

Arrows indicate positive effects and crosses indicate negative effects.