The Novel Cyclophilin Binding Compound, Sanglifehrin A, Disassociates $G_1$ Cell Cycle Arrest from Tolerance Induction

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T cell anergy has been demonstrated to play a role in maintaining peripheral tolerance to self Ags as well as a means by which tumors can evade immune destruction. Although the precise pathways involved in anergy induction have yet to be elucidated, it has been linked to TCR engagement in the setting of cell cycle arrest. Indeed, rapamycin, which inhibits T cell proliferation in $G_1$, has the ability to promote tolerance even in the presence of costimulation. To better define the role of the cell cycle in regulating anergy induction, we used the novel cyclophilin-binding ligand, sanglifehrin A (SFA). We demonstrate that SFA can inhibit TCR-induced cytokine and chemokine production without preventing TCR-induced anergy. Our data also indicate that despite its ability to induce $G_1$ arrest, SFA does not induce anergy in the presence of costimulation. Furthermore, although SFA blocks proliferation to exogenous IL-2, it does not prevent IL-2-induced reversal of anergy. When we examined the phosphorylation of 4EBP-1, a downstream substrate of the mammalian target of rapamycin, we found that rapamycin, but not SFA, inhibited the mammalian target of rapamycin activity. Based on these data, we propose that the decision as to whether TCR engagement will lead to productive activation or tolerance is dictated by a rapamycin -inhibitable pathway, independent of the $G_1\rightarrow S$ phase cell cycle progression. *The Journal of Immunology, 2004, 172: 4797–4803.

Cyclosporin A (CSA)3 and FK506 are both potent, clinically relevant immunosuppressive agents that complex within the cell to a class of proteins termed immunophilins (12). The immunophilin-drug complex blocks TCR-induced signaling by inhibiting the function of the $Ca^{2+}$ dependent phosphatase, calcineurin. Calcineurin plays a pivotal role in TCR-induced activation by facilitating the translocation of the transcription factor NF-AT to the nucleus. In as much as NF-AT activation is also critical for the induction of T cell anergy, both CSA and FK506 block tolerance induction as well as T cell activation (13). Rapamycin is another macrolide immunosuppressant that, like FK506, binds to the intracellular immunophilin FK506 binding protein (14). However, unlike FK506 and CSA, rapamycin does not inhibit TCR-induced calcineurin activity. Rather, the rapamycin -FKBP complex serves to block T cell proliferation at $G_1$ by inhibiting the activity of the kinase mammalian target of rapamycin (mTOR). Thus, while FK506 and CSA exert their inhibitory effects by blocking TCR-induced activation, rapamycin blocks distally at the level of the response to cytokines/growth factors. As a result, unlike CSA and FK506, rapamycin does not inhibit TCR-induced anergy. On the contrary, rapamycin has the ability to promote anergy induction even in the presence of costimulation (8).

Recently, a novel immunophilin-binding compound, sanglifehrin A (SFA), has been reported to have immunosuppressive properties (15, 16). The exact biochemical and molecular mechanisms accounting for SFA’s ability to suppress T cell function have yet to be elucidated. As is the case for CSA, SFA forms intracellular complexes with cyclophilin. In fact, SFA was originally isolated using a cyclophilin affinity column (17, 18). However, SFA does not inhibit calcineurin activity. Rather, similar to rapamycin, SFA exerts its immunosuppressive effects by blocking T cell proliferation in $G_1$. In light of the fact that the induction of T cell anergy has been linked to TCR engagement in the absence of cell cycle

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3 Abbreviations used in this paper: CSA, cyclosporin A; SFA, sanglifehrin A; PCC, pigeon cytochrome c; mTOR, mammalian target of rapamycin; PI, propidium iodide.
progression, we were interested in determining the effect of SFA on the induction of T cell tolerance. In this report, we demonstrate that unlike CSA, SFA does not inhibit TCR-induced anergy. In contrast, although like rapamycin it inhibits proliferation at G1, SFA does not promote anergy nor prevent its reversal by IL-2. Thus, the prevention and reversal of anergy are not necessarily linked to the cell cycle but rather to an alternate rapamycin-inhibitable pathway of IL-2 signaling.

Materials and Methods

Reagents

CSA (Calbiochem, Cambridge, MA) and rapamycin (a generous gift of Dr. S. Sehgal, Wyeth-Ayerst, Princeton, NJ) were dissolved in ethanol and then added to the cultures at the indicated doses. Sanglifehrin A (a generous gift from Dr. J. Allison, University of California, Berkeley, CA) at a concentration of 3 μg/ml was added to the cultures at the indicated doses. CSA (Calbiochem, Cambridge, MA) and rapamycin (a generous gift of Dr. R. Sedrani, Novartis, Switzerland) was dissolved in DMSO.

Cell culture

All experiments were performed using A.E7, a CD4" Th1 clone specific for the pigeon cytochrome c (PCC) peptide 81-104, which was grown and maintained as previously described (19). In general, cells were stimulated for 48 h with whole PCC and irradiated (3000 rad) B10.A splenocytes as a source of APCs. The cells were next expanded 20:1 in 10 U/ml rIL-2 in the presence of increasing amounts of SFA and assayed for proliferation in a thymidine uptake assay. As seen in Fig. 1A, consistent with previously published results, SFA inhibited IL-2-induced proliferation in a dose dependent fashion. Importantly, the ability of SFA to inhibit proliferation was not secondary to nonspecific death as the percent of viable cells was equal in both the treated and untreated cells (data not shown). It has been reported that T cell proliferation induced by Ag receptor engagement can occur via IL-2-dependent and IL-2-independent pathways (22). A.E7 T cells were thus stimulated with plate-bound anti-CD3 and soluble anti-CD28 in the presence of increasing amounts of CSA, rapamycin, and SFA (Fig. 1B). As expected, CSA and rapamycin inhibited T cell proliferation. In addition, SFA inhibited proliferation with equivalent potency. These data suggest that SFA can block proliferation in CD4" T cells in response to both exogenous IL-2 and direct stimulation through the TCR in the presence of costimulation.

Previous reports have demonstrated the ability of SFA to block T cell proliferation in G0, the cell cycle (15). To ensure that such was the case for nonimmortalized CD4" T cells, cell cycle analysis was performed on cells activated in the presence of either rapamycin or SFA. As seen in Fig. 2, mock-stimulated cells are

Functional assays

T cells were assessed for their ability to proliferate to PCC by adding 4 × 10^5 A.E7 cells to 50 × 10^5 B10.A-irradiated splenocytes (3000 rad) and increasing doses of PCC in 96-well plates in triplicate. After 48 h of culture, the cells were pulsed with [3H]thymidine and harvested 16 h later. Proliferation was evaluated by thymidine incorporation which was determined using a Betaplate reader. For cytokine production, 200 × 10^5 A.E7 cells were stimulated overnight in 96-well plates that had been coated with 3 μg/ml anti-CD3 in the presence of 1/1000 soluble anti-CD28. Supernatant fluid was collected and assessed for IL-2 production or MIP-1α production by ELISA (Endogen, Rockford, IL.). For each sample, multiple dilutions of the supernatant fluid were assayed and the concentration was determined based upon the dilution that best fit the most linear aspect of the standard curve.

Western blot analysis

Cytoplasmic extracts were made by lysing equal numbers of cells in a lysis buffer containing 50 mM HEPES, pH 7.5, 0.5% Nonidet P-40, 5 mM EDTA, 50 mM NaCl, and 1 × protease inhibitor mixture (Sigma-Aldrich, St. Louis, MO). PAGE with a 10% gel was performed and the samples were then transferred to nitrocellulose. Western blot analysis was performed using 1 μg/ml anti-cyclin A (Ab-3; Calbiochem) or anti-4EBP-1 (Upstate Biotechnology, Lake Placid, NY). The secondary Ab consisted of an alkaline-phosphatase labeled anti-rabbit Ab (Sigma-Aldrich) (1:5000).

Results

Both rapamycin and SFA inhibit T cell proliferation in G1

The initial studies demonstrating the ability of SFA to inhibit proliferation were performed on immortalized T cell lines (15, 16). As such, we first wanted to confirm that SFA inhibits T cell proliferation in nonimmortalized CD4" TH1 clones (A.E7 T cell clone). A.E7 T cells were cultured in IL-2 in the presence of increasing amounts of SFA and assayed for proliferation in a thymidine uptake assay. As seen in Fig. 1A, consistent with previously published results, SFA inhibited IL-2-induced proliferation in a dose dependent fashion. Importantly, the ability of SFA to inhibit proliferation was not secondary to nonspecific death as the percent of viable cells was equal in both the treated and untreated cells (data not shown). It has been reported that T cell proliferation induced by Ag receptor engagement can occur via IL-2-dependent and IL-2-independent pathways (22). A.E7 T cells were thus stimulated with plate-bound anti-CD3 and soluble anti-CD28 in the presence of increasing amounts of CSA, rapamycin, and SFA (Fig. 1B). As expected, CSA and rapamycin inhibited T cell proliferation. In addition, SFA inhibited proliferation with equivalent potency. These data suggest that SFA can block proliferation in CD4" T cells in response to both exogenous IL-2 and direct stimulation through the TCR in the presence of costimulation.

Previous reports have demonstrated the ability of SFA to block T cell proliferation in G0, the cell cycle (15). To ensure that such was the case for nonimmortalized CD4" T cells, cell cycle analysis was performed on cells activated in the presence of either rapamycin or SFA. As seen in Fig. 2, mock-stimulated cells are...
93% in G1 and 0% in S phase, while upon stimulation, only 65% of the cell are in G1 and 18% are in S phase. The addition of rapamycin results in 92% of the cells in G1 with 3% in S phase. Likewise, the addition of SFA results in 91% of the cells in G1 with 4% in S phase. These data demonstrate that, as is the case with rapamycin, SFA inhibits T cell proliferation by promoting G1 arrest.

SFA inhibits TCR-induced cytokine and chemokine production

Thus far, we have been able to demonstrate that SFA is able to inhibit proliferation of CD4⁺ T cells both in response to exogenous IL-2 and TCR stimulation in the presence of costimulation. Next we wanted to determine the effect of SFA on TCR-induced cytokine production. A.E7 T cells were stimulated overnight by plate-bound anti-CD3 and soluble anti-CD28 in the presence of CSA, rapamycin, or SFA. As seen in Fig. 3A, cells stimulated with anti-CD3 alone (signal 1) fail to produce IL-2, while the addition of costimulation leads to IL-2 production. Furthermore, CSA is able to completely abolish IL-2 production at a dose as low as 200 nM, while rapamycin demonstrates no inhibitory effects. Interestingly, as seen in Fig. 3A, SFA was able to inhibit IL-2 production. These findings were surprising because previous reports using Jurkat T cells suggested that SFA inhibited proliferation independent of inhibiting TCR-induced cytokine production (15, 16). Because, IL-2 production is dependent on costimulation, it was possible that the ability of SFA to inhibit IL-2 was via the inhibition of anti-CD28 signaling. To address this question we examined the ability of SFA to inhibit MIP-1α production. In contrast to IL-2, as shown in Fig. 3B, MIP-1α levels are equivalent in the supernatant fluid derived from cells stimulated with anti-CD3 alone (signal 1) or anti-CD3 + anti CD28 (signal 1 plus 2). Nonetheless, SFA is able to inhibit the production of MIP-1α in the activated A.E7 cells. These data suggest that SFA is able to inhibit TCR-induced cytokine and chemokine production as well as proliferation.

The effect of SFA on the induction and reversal of anergy

Previously, our group and others have been able to demonstrate the induction of anergy when the TCR is engaged in the absence of cell cycle progression (7, 9–11). In particular, we have shown that stimulation with signal 1 plus 2 plus rapamycin (which blocks proliferation at G1) induces anergy, while stimulation with signal
1 plus 2 plus hydroxyurea (which blocks proliferation in S phase) does not induce anergy (9). This led us to hypothesize that the biochemical events that occur between G1 and S phase are responsible for anergy induction. To test this hypothesis, a series of experiments were performed examining the effect of SFA (which also blocks proliferation in G1 but by a different mechanism than rapamycin) on the induction of anergy.

Given the fact that both CSA and SFA block TCR-induced activation, we first wanted to determine the effect of SFA on TCR-induced anergy. A.E7 T cells were stimulated overnight with signal 1 plus 2 to induce full activation or signal 1 alone (anergy) to induce anergy in the presence or absence of SFA. The cells were then harvested washed extensively to remove Abs and drugs, and then rested for 7 days in fresh medium. Next, the cells were rechallenged with APC plus peptide and proliferation was measured to determine whether they were anergic. Importantly, SFA is only present during the overnight “induction” period and not during the rest period in fresh medium or rechallenge. As seen in Fig. 4, cells initially stimulated with signal 1 plus 2 proliferate to APC plus peptide upon rechallenge, while cells initially stimulated with signal 1 alone are hypoproliferative upon rechallenge (they are anergic). The addition of SFA to the cells stimulated with signal 1 alone did not prevent the induction of anergy in that they were still hypoproliferative upon rechallenge. These data suggest that even though SFA binds to cyclophilin and can inhibit TCR-induced cytokine production it does not inhibit the TCR-induced pathways necessary for the induction of anergy. Alternatively, the presence of SFA during the initial stimulation with signal 1 plus 2 did not promote anergy. Thus, even though SFA inhibits T cell proliferation at G1 unlike rapamycin it does not promote the induction of anergy when the TCR is engaged in the presence of costimulation.

An important property of anergic T cells is the fact that they produce markedly decreased levels of IL-2 upon rechallenge (2). To determine the effect of SFA on the ability of anergic T cells to produce IL-2 upon rechallenge, A.E7 cells were stimulated as in Fig. 4, rested, and this time rechallenged with plate-bound anti-CD3 + soluble anti-CD28. As is shown in Fig. 5A, cells initially stimulated with signal 1 plus 2 produce IL-2 upon rechallenge, while cells initially stimulated with signal 1 alone produce less IL-2 upon rechallenge. Likewise, cells stimulated with signal 1 plus 2 plus rapamycin are also anergic upon rechallenge. However, once again, the presence of SFA during the initial induction period did not promote the induction of anergy when costimulation was present. That is, despite the fact that the TCR was activated and cell cycle progression was blocked at G1, anergy was not induced. Furthermore, as shown in Fig. 5B, SFA does not inhibit rapamycin-induced anergy. This is not surprising since, unlike CSA, SFA does not block TCR-induced anergy. Nonetheless, the observations in Fig. 5B emphasize the fact that SFA is able to block TCR-induced activation while leaving TCR-induced anergy induction intact.

When anergic T cells are cultured in IL-2, they proliferate and in fact, their ability to respond to rechallenge progressively returns (6, 7). We have previously shown that rapamycin inhibits IL-2-induced reversal of anergy (8). Thus, we wanted to determine whether SFA (presumably by blocking IL-2-induced proliferation) could also prevent the reversal of anergy. T cells were anergized

![Figure 4](image1.png)  
**FIGURE 4.** SFA neither inhibits signal 1-induced anergy, nor promotes anergy in the presence of costimulation (signal 1 plus 2). Our in vitro model of tolerance follows the following experimental design: Induction overnight—rest 5–10 days—rechallenge, overnight and assay. For induction, A.E7 T cells were initially stimulated overnight with plate-bound anti-CD3 to induce anergy (An) or anti-CD3 plus anti-CD28 (signal 1 plus 2) in the presence or absence of SFA. Next, the cells were extensively washed and rested in fresh medium for 7 days. The cells were rechallenged with APC and increasing amounts of PCC for 48 h, pulsed with [3H]thymidine for an additional 16 h, and then assayed for proliferation. The figure depicts proliferation upon rechallenge. Note, SFA is only present during the induction period and not during rechallenge. These data are representative of more than three separate experiments.

![Figure 5](image2.png)  
**FIGURE 5.** SFA does not inhibit rapamycin-induced anergy. A.E7 T cells were initially stimulated overnight with plate-bound anti-CD3 plus anti-CD28 (signal 1 plus 2) in the presence or absence of SFA or rapamycin (Rapa) (Induction). Next the cells were extensively washed and rested in fresh medium for 7 days. During rechallenge, the cells were stimulated overnight with plate-bound anti-CD3 plus anti-CD28 and supernatant fluid was collected and assayed for IL-2 production by ELISA. A, T cells initially stimulated with signal 1 plus 2 plus SFA produce IL-2 upon rechallenge; they are not anergic. B, T cells stimulated with signal 1 plus 2 plus SFA plus rapamycin fail to produce IL-2 upon rechallenge; they are anergic. Note, the drugs are only present during the induction period and not during rechallenge. These data are representative of more than three separate experiments.
with overnight stimulation by plate-bound anti-CD3, the cells were
rested for 7 days in either fresh medium, IL-2, IL-2 plus rapamycin
or IL-2 plus SFA. Next the cells were extensively washed and they
were rechallenged with anti-CD3 + anti-CD28. As seen in Fig. 6,
the cells that were rested in medium produce minimal amounts of
IL-2 upon rechallenge while the cells cultured in IL-2 have begun
to regain their ability to respond. As expected, for the cells that
were cultured with IL-2 plus rapamycin (in the presence or ab-
sence of SFA) anergy was not reversed. In contrast, the cells cul-
tured in IL-2 plus SFA are regaining their ability to produce IL-2.
Thus, despite the fact that SFA prevents IL-2-induced proliferation
in G1, it does not prevent IL-2-induced anergy reversal.

Rapamycin but not SFA inhibits mTOR activity

In as much as both rapamycin and SFA inhibit T cell proliferation
in G1, it was somewhat surprising that SFA neither promoted an-
ergy in the presence of costimulation nor prevented IL-2-induced
reversal of anergy. Thus, we were interested in determining bio-
chemical differences that might account for these observations.
IL-2R engagement leads to the activation of mTOR and the sub-
sequent progression of the cell cycle. Rapamycin blocks T cell
proliferation in G1 by inhibiting mTOR (23). As such, experiments
were initiated to determine whether SFA also inhibits mTOR. T
cells were either mock-stimulated or stimulated with anti-CD3
plus anti-CD28 in the presence or absence of either rapamycin or
SFA. The cells were next lysed and Western blot analysis was
performed on cytoplasmic extracts. Stimulation overnight with sig-
nal 1 plus 2 led to the up-regulation of cyclin A (Fig. 7, lane 2). As
seen in lanes 3 and 4, both rapamycin and SFA have the ability to
block this up-regulation. This was expected because the up-regu-
lation of cyclin A occurs as the cells move from G1 to S phase.
Next, we used the same extracts to examine mTOR activity. 4E-
BP1 is phosphorylated by mTOR and the phosphorylated protein
migrates slower than its unphosphorylated form (24). Thus, the
phosphorylation status of 4EBP-1 may be used as an indicator of
mTOR activity. As seen in Fig. 7, stimulation with signal 1 plus 2
leads to the hyperphosphorylation of 4EBP-1 indicative of mTOR
activation (as seen by an increase in the band denoted by the upper
arrow). The presence of rapamycin inhibits 4EBP-1 phosphoryla-
tion consistent with the fact that rapamycin inhibits mTOR activity
(note the presence of the unphosphorylated band denoted by the
lower arrow). However, 4EBP-1 is hyperphosphorylated in the

Discussion

The ultimate outcome of Ag recognition by T cells is determined
by the context in which the TCR is engaged. In the inflammatory
milieu, in the presence of danger signals, Ag is presented by ac-
tivated APCs in the context of costimulation resulting in T cell
activation (25). In contrast, when the same Ag is presented by a
resting APC, tolerance in the form of peripheral T cell deletion or
anergy ensues. Several groups have been able to demonstrate an-
ergy to self Ag as a means of maintaining peripheral tolerance in
vivo (26–29) Furthermore, T cell anergy has been shown to be an
important mechanism by which tumors can evade immune destruc-
tion (30–33). More recently, there is evidence to suggest that not
only are anergic T cells hyporesponsive but in some cases possess
T regulatory cell function, thus enabling them to further promote
tolerance (34).

The link between cell cycle arrest and anergy induction has been
demonstrated both in vitro and in vivo (9–11, 35–38). It has been
proposed that the up-regulation of the cell cycle inhibitors, p27 and
p21, in anergic cells do not merely provide markers for hypores-
sponsive cells, but actually play an important role in maintaining
the anergic state (10, 37). In contrast, our group has proposed that
TCR engagement in the absence of cell cycle progression leads to
anergy independent of p27 (9). This is based on our findings that
T cells anergized in the presence of CSA maintain high levels of
p27 even though anergy induction is blocked and the observation
that T cells from p27, p21 and double knockout mice are readily
anergized (Ref. 9; J. D. Powell, unpublished observations). We
hypothesized that the biochemical events which account for the
G1→S phase transition are also responsible for promoting full T
cell activation.

Surprisingly, despite the fact that SFA blocks T cell proliferation
in G1, cells stimulated in the presence of SFA were not ren-
dered anergic. In contrast, rapamycin, which also blocks prolif-
model is consistent with the recent observations of Colombetti et al. (39). Although our data demonstrate that T cells blocked in G1 can still fully respond upon rechallenge, this group has demonstrated that T cell anergy can be maintained even in proliferating cells. They conclude that the reversal of anergy requires an IL-2 dependent, rapamycin -sensitive pathway that is independent of cell cycle progression (39). Indeed, we would propose that the PI-3-like kinase mTOR itself plays a central role in determining the decision between activation and tolerance. In many cell types, mTOR regulates cell size and proliferation by integrating cues from the environment in terms of the availability of nutrients (40). Similarly, in T cells, mTOR kinase might regulate T cell activation by integrating cues from the environment in terms of the presence or absence of danger.

Our data demonstrate the ability of SFA to block T cell proliferation in nonimmortalized CD4+ T cells. In addition, we demonstrate that SFA also has the ability to inhibit TCR-induced cytokine and chemokine production. These latter observations are in contrast to two other studies that concluded that SFA blocks proliferation independent of cytokine production (15, 16). Importantly, these other reports based their conclusions on experiments involving the immortalized Jurkat T cell line. In addition, PMA was used as a stimulus that is able to activate T cells in a manner that bypasses the necessity for TCR engagement. In contrast, our studies were performed using nonimmortalized, Ag-specific CD4+ T cell clones stimulated through the TCR in the presence of costimulation. The precise mechanism by which SFA mediates this effect is not clear. Importantly, this does not appear to be a non-specific effect in that although SFA inhibited TCR-induced IL-2 and MIP-1α production, TCR-induced anergy remained intact. This is consistent with the findings that although SFA was originally defined as a ligand for cyclophilin, it does not block calcineurin activity (18). Recently, Zhang et al. (41) have shown that SFA is able to activate p53 at the level of transcription. In this regard, p53 has been shown to have the ability to repress IL-2 production in activated T cells (42). Thus, it is possible that SFA-mediated inhibition of IL-2 production is by a p53-dependent mechanism.

In addition to blocking T cell activation, SFA has recently been shown to potently inhibit the production of IL-12 by dendritic cells (43). Also, the fact that SFA and rapamycin work by different mechanisms (14, 15) and their insulin-like growth factor (IGF)-1 receptor (IGF-1R) activity (19). Recently, Zhang et al. (41) have shown that SFA is able to activate p53 at the level of transcription. In this regard, p53 has been shown to have the ability to repress IL-2 production in activated T cells (42). Thus, it is possible that SFA-mediated inhibition of IL-2 production is by a p53-dependent mechanism.

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References

1. Mueller, D. L., and M. K. Jenkins. 1995. Molecular mechanisms underlying functional T-cell unresponsiveness. Curr. Opin. Immunol. 7:375.
2. Powell, J. D., J. A. Ragheb, S. Katagawa-Sakakida, and R. H. Schwartz. 1998. Molecular regulation of interleukin-2 expression by CD28 co-stimulation and anergy. Immunol. Rev. 165:267.
31. Sotomayor, E. M., I. Borrello, E. Tubb, J. P. Allison, and H. I. Levitsky. 1999. In vivo blockade of CTLA-4 enhances the priming of responsive T cells but fails to prevent the induction of tumor antigen-specific tolerance. Proc. Natl. Acad. Sci. USA 96:14476.

32. Reilly, R. T., M. B. Gottlieb, A. M. Ercolini, J. P. Machiels, C. E. Kane, F. I. Okoye, W. J. Muller, K. H. Dixon, and E. M. Jaffe. 2000. HER-2/neu is a tumor rejection target in tolerized HER-2/neu transgenic mice. Cancer Res. 60:3569.

33. Ye, X., J. McCarrick, L. Jewett, and B. B. Knowles. 1994. Timely immunization subverts the development of peripheral nonresponsiveness and suppresses tumor development in simian virus 40 tumor antigen-transgenic mice. Proc. Natl. Acad. Sci. USA 91:3916.

34. Chai, J. G., J. Y. Tsang, R. Lechler, E. Simpson, J. Dyson, and D. Scott. 2002. CD4<sup>+</sup>CD25<sup>+</sup> T cells as immunoregulatory T cells in vitro. Eur. J. Immunol. 32:2365.

35. Greenwald, R. J., V. A. Boussiotis, R. B. Lorsbach, A. K. Abbas, and A. H. Sharpe. 2001. CTLA-4 regulates induction of anergy in vivo. Immunity 14:145.

36. Wells, A. D., M. C. Walsh, J. A. Bluestone, and L. A. Turka. 2001. Signaling through CD28 and CTLA-4 controls two distinct forms of T cell anergy. J. Clin. Invest. 108:893.

37. Jackson, S. K., A. DeLoose, and K. M. Gilbert. 2001. Induction of anergy in Th1 cells associated with increased levels of cyclin-dependent kinase inhibitors p21<sup>CIP1</sup> and p27<sup>KIP1</sup>. J. Immunol. 166:952.

38. Vanasik, T. L., A. Khoruts, T. Zell, and D. L. Mueller. 2001. Antagonistic roles for CTLA-4 and the mammalian target of rapamycin in the regulation of clonal anergy: enhanced cell cycle progression promotes recall antigen responsiveness. J. Immunol. 167:5636.

39. Colombetti, S., F. Benigni, V. Basso, and A. Mondino. 2002. Clonal anergy is maintained independently of T cell proliferation. J. Immunol. 169:6178.

40. Kim, D. H., D. D. Sarbasov, S. M. Ali, J. E. King, R. R. Latke, H. Erdjument-Bromage, P. Tempst, and D. M. Sabatini. 2002. mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. Cell 110:163.

41. Zhang, L. H., H. D. Youn, and J. O. Liu. 2001. Inhibition of cell cycle progression by the novel cyclophilin ligand sanglifehrin A is mediated through the NF-κB-dependent activation of p53. J. Biol. Chem. 276:45324.

42. Chaudhry, S., W. J. Freebern, J. L. Smith, W. G. Butscher, C. M. Haggerty, and K. Gardner. 2002. Cross-regulation of T cell growth factor expression by p53 and the Tax oncogene. J. Immunol. 169:6767.

43. Steinschulte, C., T. Taner, A. W. Thomson, G. Bein, and H. Hackstein. 2003. Sanglifehrin A, a novel cyclophilin-binding immunosuppressant blocks bioactive IL-12 production by human dendritic cells. J. Immunol. 171:542.