Rapamycin Improves Lymphoproliferative Disease in Murine Autoimmune Lymphoproliferative Syndrome (ALPS)

Authors: David T. Teachey,¹,² Dana A. Obzut,¹ Kelly Axsom,¹ John K. Choi,⁴ Kelly C. Goldsmith,¹ Junior Hall,¹ Jessica Hulitt,¹ Catherine Manno,² John M. Maris,¹ Nicholas Rhodin,¹ Kathleen Sullivan,³ Valerie I. Brown,¹ and Stephan A. Grupp.¹,⁴

Affiliations: Divisions of ¹Oncology, ²Hematology and ³Immunology in the Department of Pediatrics, and ⁴Department of Pathology and Laboratory Medicine, Children’s Hospital of Philadelphia, University of Pennsylvania School of Medicine, Philadelphia, PA 19104

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Corresponding Author: David T. Teachey, MD. Divisions of Oncology and Hematology, Children’s Hospital of Philadelphia, ARC 902, 3615 Civic Center Boulevard, Philadelphia, PA 19104. Email: teacheyd@email.chop.edu
Abstract

Autoimmune Lymphoproliferative Syndrome (ALPS) is a disorder of abnormal lymphocyte survival caused by defective Fas-mediated apoptosis, leading to lymphadenopathy, hepatosplenomegaly, and an increased number of double negative T cells (DNTs). Treatment options for ALPS patients are limited. Rapamycin has been shown to induce apoptosis in normal and malignant lymphocytes. Since ALPS is caused by defective lymphocyte apoptosis, we hypothesized rapamycin would be effective in treating ALPS. We tested this hypothesis using rapamycin in murine models of ALPS. We followed treatment response with serial assessment of DNTs by flow cytometry in blood and lymphoid tissue, by serial monitoring of lymph node and spleen size with ultrasonography, and by ELISA for anti-dsDNA antibodies. Three-dimensional ultrasound measurements in the mice correlated to actual tissue measurements at sacrifice (r=0.9648). We found a dramatic and statistically significant decrease in DNTs, lymphadenopathy, splenomegaly, and autoantibodies after only 4 weeks when comparing rapamycin treated mice to control. Rapamycin induced apoptosis through the intrinsic mitochondrial pathway. We compared rapamycin to mycophenolate mofetil, a second-line agent used to treat ALPS, and found rapamycin’s control of lymphoproliferation was superior. We conclude that rapamycin is an effective treatment for murine ALPS and should be explored as treatment for affected humans.
Introduction

Patients with ALPS typically present in early childhood with lymphadenopathy and organomegaly and many patients develop autoimmune cytopenias and secondary neoplasms. ALPS is thought to be a rare condition,\(^1\) however, we have demonstrated in a single institution study that ALPS is likely more common than previously thought.\(^2\) Treatments for patients with ALPS are limited to non-specific immune modulators with extensive side effect profiles.\(^3\) The pathophysiology of ALPS has been partially characterized allowing for the rational evaluation of potential treatments.

ALPS is caused by defects in the Fas apoptotic pathway, suggesting agents that can induce lymphocyte apoptosis may be useful treatments. Rapamycin is an immunosuppressive and anti-neoplastic agent with a tolerable side effect profile, belonging to a class of drugs that inhibit the mammalian target of rapamycin (mTOR).\(^4\) These mTOR inhibitors may induce apoptosis in T and B lymphocytes.\(^5\)\(^,\)\(^6\) We hypothesized that rapamycin would be effective in reducing symptoms and treating the disease in patients with ALPS by inducing apoptosis in abnormal B and T lymphocytes. Rapamycin has been studied in other illnesses and in pediatric patients,\(^4\) however, prior to testing any novel therapy in ALPS patients, it is important to establish efficacy in animal models.

The majority of patients with ALPS have mutations in the Fas gene.\(^7\) Multiple murine models with different backgrounds exist with Fas mutations, however, CBA-\(lpr^{fg}\) has been shown to have a phenotype and genotype most similar to the majority of patients with ALPS.\(^1\) We studied the efficacy of rapamycin in CBA-\(lpr^{fg}\) mice and established the activity of this agent against ALPS. We also studied the efficacy of
rapamycin against autoimmunity using a similar mouse model, MRL-lpr. We used high frequency small animal ultrasonography to follow response to drug and this is the first report, we are aware of, to use ultrasound to follow a non-malignant murine disease.

**Materials and Methods**

*Animals and Rapamycin Treatment Schedule*

CBA-\textit{lpr} \textsuperscript{58} mice 5-6 month old (Jackson Laboratories, Bar Harbor, ME) were randomized (1:1) to treatment with rapamycin (Wyeth Ayerst Laboratories, Marietta, PA) or vehicle after establishment of clinically identifiable lymphadenopathy, defined as having at least one palpable lymph node greater than 100mm\textsuperscript{3} in size as assessed by high frequency small animal ultrasonography (see below). Rapamycin was given at a dose of 5mg/kg/day, 5 days a week.\textsuperscript{6} Mice were treated in 2 cohorts. The first cohort of 4 mice (2 treatment: 2 control) was treated for 6 weeks and sacrificed for tissue analysis. In the second cohort, 11 mice (6 treatment: 5 control) were treated for 3 months and then sacrificed.

*Assessment of Response to Rapamycin Treatment: Ultrasound Measurement of Lymph Node and Spleen Size*

High frequency small animal ultrasonography is as a novel tool to measure changes in organ or tumor size with disease progression or in response to drug treatment.\textsuperscript{8} Ultrasound imaging was performed using a Vevo 660 small animal ultrasound (VisualSonics, Inc, Toronto, Ontario, Canada). This model ultrasound was recently demonstrated to be reproducibly accurate in 3-D assessment of intra-abdominal tumor
size in a murine pancreatic cancer model.\textsuperscript{8} We used the Vevo 660 to follow lymph node volume and splenic area. Prior to using the ultrasound to follow disease burden in these experiments, measurements from our machine were validated by comparing measured organ sizes to actual sizes by serial sacrifice of multiple strains of mice and evaluation of multiple tissues (Rhodin, et. al., unpublished data).

Prior to the day of ultrasound abdominal hair was removed from the mice using a depilatory cream. Mice were anesthetized using isofluorane. We used a 40 MHz center frequency probe for our measurements that has a fixed focal depth of 6mm and resolution to 30 microns. B-mode imaging was used to obtain two dimensional dynamic images of mouse lymph nodes and spleens. 3-D images were collected of lymph nodes by a motorized drive that collects regular spatial images through a tissue at 30 $\mu$m slices. Data were stored and analyzed using Visualsonics software version 1.3.8.

Mice were randomized to rapamycin or vehicle after they developed at least one palpable lymph node. Ultrasound assessment of lymph node size was performed the day mice were enrolled onto study and started drug treatment. Only mice with at least one lymph node of 100mm\textsuperscript{3} in volume as assessed by ultrasound were enrolled and we followed the largest lymph node at initiation of treatment in each mouse. Upon study entry and every two weeks until sacrifice, one lymph node was serially measured for volume by 3-D imaging and splenic length and height were measured by B-mode imaging. Comparisons were made between treated and untreated animals by two-sided t test. We compared actual size of spleen and lymph nodes by caliper measurements to ultrasound measurements at sacrifice to validate measurements. All data analysis and ultrasound measurements were blinded as to treatment arm.
Assessment of Response to Rapamycin Treatment: Peripheral Blood for DNTs

Patients and mice with ALPS have an increased number of an unusual T cell population, double negative T cells (DNTs, T cell phenotype CD3+, CD4-, CD8−, TCRαβ+) in both peripheral blood and lymphoid tissues. We followed blood counts and DNTs in the mice by retro-orbital bleed at initiation of treatment and serially every other week. Comparisons were made between treated and untreated by two-sided t test. CBC analysis was performed on a HemeVet 850FS hematology analyzer (CDC Technologies, Oxford, CT) and flow cytometric analysis for DNTs was performed using anti-TCR B chain-FITC, anti-CD4-PE, anti-CD8a (Ly-2)-PerCP-CY5.5, and anti-CD3ε-APC-Cy7. All antibodies were purchased from BD Pharmigen (Franklin, NJ). We monitored absolute double negative T cell count, defined as WBC (measured by HemeVet hematology analyzer) x % lymphocytes (calculated by HemeVet hematology analyzer and confirmed by FSC/SSC on flow cytometry and manual differential on peripheral smear) x % DNTs (calculated by flow cytometry).

Comparison of Rapamycin to Mycophenolate Mofetil

Mycophenolate Mofetil (MMF) is an immunosuppressant that has been used to treat ALPS patients. We compared the activity of rapamycin to MMF in our murine ALPS model. We randomized mice to 2:2:1 with rapamycin, MMF, or vehicle control after development of identifiable lymphadenopathy as described in Treatment Schedule above. 5 mice were treated with rapamycin at 5mg/kg/day and 5 mice were treated with MMF (F. Hoffmann-La Roche, Basal, Switzerland) at 100mg/kg/day I.P. 9 mice
received no treatment, representing a cohort from this randomization plus historical controls from the earlier experiments described in this work. Mice were treated for 3 months and lymphadenopathy, splenomegaly, and peripheral blood DNTs were followed serially as described above.

Assessment of autoantibodies

MRL-\textit{lpr} mice (Jackson Laboratories, Bar Harbor, ME) were randomized (1:1:1) to treatment with rapamycin, MMF, or vehicle control beginning at 3 months of age. 4 mice were treated with rapamycin at a dose of 5mg/kg/day by gavage, 4 mice were treated with MMF at a dose of 100mg/kg/day I.P., and 4 mice received no treatment. Mice were treated for 4 weeks and serum was obtained from mice by retro-orbital bleed at initiation of treatment and serially every other week. Serum samples were frozen at collection and all samples were analyzed together for mouse anti-ds DNA IgG specific antibodies using a 96 well quantitative ELISA kit from Alpha Diagnostic, International (San Antonio, TX). All samples were run in duplicate as recommended in the manufacturer’s instructions and 1ul of sera was used for each well.

Immunoblotting

Immunoblots were performed to detect the mTOR pathway target S6, in order to assess inhibition of this pathway, and to detect the phosphorylation of the pro-apoptotic molecule BAD. For these experiments, we used our published methods\textsuperscript{13} based on a modification of the protocol of Huang, et al.\textsuperscript{14} In brief, mouse lymph nodes were harvested and dissociated into a single-cell. 10-20 x 10\textsuperscript{6} cells were lysed in ice cold lysis
buffer and nuclei were sedimented from the lysate. Equal quantities of protein (20-40 mcg) from each lysate were resolved using NuPAGE Tris-bis SDS gel electrophoresis (10% SDS for S6 and 12% SDS for BAD) (Cell Signaling Technologies), transferred to PVDF membranes (PerkinElmer Life Sciences, Boston, MA), and labeled with antibodies to S6, phospho-S6 (ser235/ser236), BAD, and phospho-BAD (Ser 112 and Ser 136) (Cell Signaling Technologies) for 18 hours at 4°C and detected using an HRP-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch laboratories, West Grove, PA) and the Lumiglo chemiluminescence system (Cell Signaling Technologies). Bands were outlined and quantitated densitometrically using Quantity-One software (BioRad).

**In vitro assessment of apoptosis**

T lymphocytes from CBA-Lpr/lpr mice were sustained in vitro for apoptosis testing using published techniques. In brief, lymph nodes were harvested from 5-6 month old mice. Cells were maintained at 37°C under a 5% CO₂ in RPMI 1640 media with 10% heat inactivated fetal calf serum, 10 mM Hepes (pH 7.5), 1 mM sodium pyruvate, 100 µM nonessential amino acids, 100 units/ml penicillin, 100 µg/ml streptomycin, 50 µM 2-mercaptoethanol (Fisher Scientific), and 10U/ml murine IL-2 (Lenico, St. Louis, MO). After 7 days in culture, viable cells were isolated by Ficoll-Isopaque and expanded. This methodology can successfully support and maintain CD3+4+ and CD3+8+ T lymphocytes from the mice. Double negative T cells do not survive in culture. Seven days later, aliquots of 10⁵ lymphocytes were exposed to 100ng/ml rapamycin for 48 hours and were assessed for apoptosis by flow cytometric staining for annexin-V-FITC (BD Pharmigen) and 7-aminoactinomycin-D (7-AAD, BD Pharmigen). Apoptotic cells are
annexin-V positive and 7-AAD negative. In order to determine if apoptosis was caspase dependent, prior to treating with rapamycin, aliquots of cells were pretreated for 2 hours with 50uM pancaspase inhibitor (Z-VAD-FMK), 50 uM caspase-9 inhibitor (Z-LEHD-FMK), or 50uM Caspase-8 Inhibitor (Z-IETD-FMK). Caspase inhibitors were all purchased from Biovision Research Products (Mountain View, CA). Cells were also treated with 5nM etoposide (Sicor, Irvine, CA) as a positive control for apoptosis.

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**Figure 1. Rapamycin decreases lymphoproliferation.** CBA-\(lpr^{cs}\) mice were randomized to treatment with rapamycin vs vehicle control. After 4 weeks of treatment a visually apparent decrease in adenopathy is appreciated.

**Results**

*Rapamycin Decreases Lymphoproliferation in Murine ALPS*
We found a striking decrease in lymphoproliferation in the mice after treatment with rapamycin (Figure 1). At initiation of treatment, average size of lymph nodes and spleen were not statistically different between treated and control group as assessed by ultrasound measurement (Figure 2). As early as 2 weeks after initiating treatment, rapamycin treated mice had a statistically significant decrease in lymph node size (Figure 2) and by 4 weeks after initiating treatment, rapamycin treated mice had a statistically significant decrease in spleen size (Figure 2). We compared actual size of lymph nodes (Figure 3, correlation coefficient \( r = 0.96, p = 0.0001 \)) and spleen (correlation coefficient \( r = 0.94, p < 0.001 \)) to ultrasound measurements obtained on day of sacrifice and found correlation between measurements. These results demonstrate that rapamycin decreases both lymphadenopathy and splenomegaly in murine ALPS and that small animal ultrasound provides an accurate tool to measure lymphoproliferation in mice.

**Figure 3. Ultrasound accurately estimates organ volume.** In order to ensure ultrasound measurements reflected actual measurements, we compared lymph node volumes from CBA-\( lpr^{K} \) mice at sacrifice [calculated as \( 4/3\pi * \text{radius height} * \text{radius width} * \text{radius length of lymph node using caliper} \)] to volume calculated on Vevo 660 ultrasound. Linear regression analysis demonstrated a statistically significant correlation.
Rapamycin Decreases DNTs in Murine ALPS

Patients with ALPS have an increased number of an atypical T cell population, double negative T cells (DNTs, T cell phenotype CD3+, CD4-, CD8-, TCRαβ+), that represent less than 1% of the T cell population in normal individuals. DNTs are...
elevated in both peripheral blood and lymphoid tissues in patients with ALPS. These cells express both the T cell receptor and CD3; however, lack expression of CD4 and CD8. These cells may represent CD8+ cytotoxic T lymphocytes which lose CD8 expression or a subset of regulatory T (Treg) cells.

CBA-lpr^c^ mice also develop elevated DNTs in blood and lymphoid tissue. We followed blood counts and DNTs in the mice by serial sampling of peripheral blood and found a statistically significant decrease in peripheral blood absolute DNTs by 4 weeks of treatment with rapamycin (Figure 4). We found the decrease in DNTs was durable even in a cohort treated for 3 months.

This decrease in DNTs was also observed in lymphoid tissues, including spleens [average number in treated, $57 \times 10^6$ (range $30 - 73 \times 10^6$) versus control, $158 \times 10^6$ (range $48 - 345 \times 10^6$) ($p = 0.04$)] and lymph nodes [average number in treated, $7 \times 10^6$ (range $0 - 27 \times 10^6$) versus control, $142 \times 10^6$ (range $67 - 316 \times 10^6$) ($p=0.009$)].

These results demonstrate that rapamycin decreases DNTs, a surrogate marker of disease in murine ALPS.
Toxicity Profile of Rapamycin in Murine ALPS

No toxicities were noted in mice treated with rapamycin. No difference was noted in hemoglobin or platelet count between groups and no mice developed anemia or thrombocytopenia. No mouse developed any apparent infection or other clinical manifestation of immunosuppression during treatment with rapamycin. The percentage of CD3/4+ and CD3/8+ T cells increased in the lymph nodes of rapamycin treated animals as the percentage of double negative T cells decreased. Because there was a significant reduction in size and cell number of lymph node and spleen in treated animals, absolute CD3/CD4 counts in lymph node and spleen were reduced in treated animals (lymph node: treated average 15 x 10^6; untreated control average 41 x 10^6, p = 0.03; spleen: treated average 28 x 10^6; untreated control average 48 x 10^6, p = 0.04). Absolute CD3/CD8 counts were also reduced, however, the reduction was not statistically significant.

Rapamycin Superior to Mycophenolate Mofetil in Murine ALPS

Mycophenolate mofetil (MMF) has been proposed as a treatment for patients with ALPS. We therefore compared treatment with rapamycin to MMF in these CBA-\textsuperscript{Ipr}\textsuperscript{sg} mice. We found rapamycin treatment resulted in superior responses against lymphoproliferation as compared to MMF. As we found in our earlier experiments, rapamycin treated mice had a statistically significant decrease in lymphadenopathy, splenomegaly and DNTs (data not shown). Mice treated with MMF had disease stabilization only for 4-6 weeks followed by progression. (Figure 5 and Supplemental
Mice treated with MMF did have an initial trend toward reduction in DNTs ($p = 0.07$) when compared to control at 6 weeks; however, mice then progressed (Supplemental Figure 2). When comparing rapamycin with MMF we found a statistically significant reduction in lymphadenopathy ($p = 0.04$) and splenomegaly ($p = 0.02$) after 6 weeks in the rapamycin treated mice (Figure 5 and Supplemental Figure 2). We found rapamycin reduced DNTs to a greater degree than MMF; however, the difference was not statistically significant (Supplemental Figure 2). These data indicate that rapamycin was superior to MMF in this murine model of ALPS.

**Figure 5. Rapamycin is superior to mycophenolate mofetil (MMF).** CBA-*$lpr^S$ mice were randomizd to treatment with rapamycin, MMF, or control. Serial ultrasounds were performed every 2 weeks to document lymph node volume in mm$^3$. Rapamycin treated mice showed a statistically significant ($p = 0.04$) decrease in lymph node volume after 6 weeks of treatment when compared to MMF treated mice. Bars represent mean lymph node volume from mice at each timepoint and error bars represent SEM. $p$ values depict comparisons of rapamycin and MMF treatment by two-tailed t-test.
Assessment of Autoantibodies

Although the CBA-lpr<sup>c9</sup> mouse is felt to be the most similar to human ALPS, these mice only develop minimal autoantibodies. We used a different murine ALPS model, MRL-lpr to assess the effect of rapamycin and MMF on murine autoantibodies. We measured mouse anti-dsDNA IgG specific antibodies in the mice using a quantitative ELISA. We found rapamycin substantially reduced the level of autoantibodies in the mice after only 2 weeks of treatment (Figure 6).

While there was no statistical difference between titer levels in the three groups at initiation of treatment, we found a statistically significant difference in average titer levels comparing rapamycin to MMF (0.3ug/ml vs 5ug/ml, \( p = 0.001 \)) and rapamycin to control (0.3ug/ml vs 8ug/ml, \( p = 0.001 \)) after 4 weeks of treatment. We found MMF did not decrease antibody levels.

**Figure 6.** Rapamycin decreases autoantibody production. MRL-lpr mice were randomized to treatment with rapamycin, MMF, or control. Retro-orbital bleeds were obtained every two weeks to measure mouse anti-dsDNA IgG specific antibodies in sera by quantitative ELISA. Mice treated with rapamycin had a statistically significant decrease in average titer levels compared to MMF (0.3ug/ml vs 5ug/ml, \( p = 0.001 \)) and control (0.3ug/ml vs 8ug/ml, \( p = 0.001 \)) after 4 weeks of treatment. Figure 6 depicts results of antibody titers for each mouse over time with 4 mice in each treatment group. Results are normalized to titer at initiation of treatment for each mouse. Error bars represent standard error of mean.
production, but did stabilize levels in some of the animals, consistent with other studies that demonstrated MMF has little effect on autoantibodies in MRL-\(lpr\) mice.\textsuperscript{19}

\textit{Evaluation of mTOR Signaling Pathway by Immunoblot}

We hypothesized the clinical response to rapamycin would be associated with a biochemical response, indicating downregulation of the mTOR signaling pathway. We have previously shown that activation of S6K1 (p70S6 kinase), an mTOR pathway intermediate, is downregulated by rapamycin in murine B cell lines.\textsuperscript{6} Similarly, other groups have assessed phosphorylation of S6, the target of S6K1, in normal cells as a marker of mTOR inhibition.\textsuperscript{20} When mTOR is active, S6K1 phosphorylates S6, leading to ribosomal protein synthesis. Inhibition of mTOR results in dephosphorylation of S6 and inhibits this process. We performed immunoblots on lymph node cells obtained from treated and control mice to assess phosphorylation of S6. In order to compare treated to untreated cells in the same mouse, we also removed a lymph node from a mouse prior to rapamycin treatment and removed a second lymph node from the same mouse after 3 of treatment and assessed phosphorylation of S6. We were able to show downregulation of the mTOR pathway as a consequence of rapamycin treatment (\textbf{Figure 7}). Immunoblot analysis of cell lysates from rapamycin treated mice demonstrated a 40 to 62\% decrease in the amount of phospho-S6 compared to cells from untreated mice. These results demonstrate that the clinical response we found in the mice is associated with a biochemical response in the mTOR signaling pathway.
Figure 7. Mechanism of action of rapamycin in ALPS.

7a. Rapamycin downregulates phospho-S6. Lymph node cells were harvested from a mouse (#1) both before (pre-tx) and after (post-tx) treatment with rapamycin for 3 days. Lymph node cells were also harvested from a mouse treated with rapamycin for 4 weeks (#3, long tx) compared to a mouse treated with vehicle for four weeks (#2, untx). Immunoblot of phospho-S6 (ser235/236) (top bands), total S6 (middle bands), and \( \beta \) tubulin (bottom bands) from the mice demonstrates a correlation between biochemical and clinical response to rapamycin in the mice. Lymph cells from treated mice had downregulation of phospho-S6 compared to lymph cells from untreated mice.

7b. Rapamycin downregulates phospho-BAD. Lymph node cells were harvested from a mouse treated with rapamycin for 4 weeks and a mouse treated with vehicle for 4 weeks. Immunoblot of phospho-BAD (Ser112) (top bands), total BAD (middle bands), and \( \beta \) tubulin (bottom bands) from the mice demonstrates downregulation phospho-BAD in the rapamycin treated cells. Results for phospho-BAD (Ser 136) were similar and are not shown.

7c. Rapamycin induces caspase dependent apoptosis in cultured murine ALPS lymphocytes. T cells from lymph node biopsy from CBA-Ipr<sup>++</sup> mice were maintained in culture. Aliquots of \( 10^5 \) lymphocytes were exposed to 100ng/ml rapamycin for 48 hours and were assessed for apoptosis by flow cytometric staining for Annexin-V. In addition, prior to treating with rapamycin, aliquots of cells were pretreated for 2 hours with a pancaspase inhibitor, a caspase-9 inhibitor, or a caspase-8 Inhibitor. Figure 7c depicts percentage of apoptotic cells (Annexin-V positive/7-AAD negative) cells by flow cytometric analysis.

Assessment of Mechanism of Response to Rapamycin

We hypothesized rapamycin would be effective in ALPS by inducing apoptosis in abnormal T lymphocytes. We examined lymphocyte apoptosis in vivo by assessing phosphorylation status of the pro-apoptotic molecule BAD and we examined lymphocyte
apoptosis in vitro by flow cytometric analysis for Annexin-V of rapamycin treated lymphocytes derived from CBA-lpr<sup>cg</sup> mice. Further, we pretreated the lymphocytes with 3 different caspase inhibitors to determine if the apoptosis was caspase-dependent and whether the apoptosis was intrinsic to the mitochondrial apoptotic pathway.

BAD is a Bcl2 family, pro-apoptotic molecule that is regulated by phosphorylation. When BAD is phosphorylated it is then sequestered by 14-3-3 proteins and rendered inactive, providing a survival signal to cells. In contrast, when BAD is dephosphorylated it provides pro-apoptotic effects on cells through the intrinsic mitochondrial pathway. Prior work has shown that mTOR inhibitors, including rapamycin, lead to the desphosphorylation of BAD with subsequent apoptosis. We harvested lymph node cells from CBA-lpr<sup>cg</sup> mice treated with rapamycin and control mice in order to assess phosphorylation of BAD. We were able to show decreased phosphorylated BAD in rapamycin treated mice (Figure 7), suggesting activation of the intrinsic mitochondrial pathway. Immunoblot analysis of cell lysates from rapamycin treated mice demonstrated a greater than 80% reduction in phospho-BAD compared to cells from treated mice.

We also treated cultured T lymphocytes obtained from harvested mouse lymph nodes with rapamycin. We found rapamycin induced apoptosis in the cultured T lymphocytes and this apoptosis could be significantly but not completely blocked with the addition of a caspase-9 or pan-caspase inhibitor, but could not be blocked with the addition of a caspase-8 inhibitor (Figure 7). These results suggest rapamycin works, at least in part, through a caspase-dependent apoptotic mechanism and primarily targets the intrinsic mitochondrial apoptotic pathway.
Discussion

Rapamycin, an mTOR inhibitor, is both an immunosuppressant and anti-neoplastic agent. Rapamycin is FDA approved as an immunosuppressive agent in solid organ transplantation. It has documented efficacy in autoimmune diseases, including rheumatoid arthritis. Rapamycin has been shown to induce apoptosis in B and T lymphocytes. mTOR inhibitors are active in models of EBV lymphoproliferative disease, and we have demonstrated that rapamycin and other drugs that target the mTOR pathway are effective against lymphoid malignancies. Since ALPS is caused by defective lymphocyte apoptosis, we hypothesized rapamycin would be an effective agent in ALPS by inducing apoptosis in the defective lymphocytes. We demonstrated rapamycin is very effective in reducing disease burden in murine ALPS and we have evidence that suggests the mechanism of that response was through caspase-dependent apoptotic induction through the intrinsic mitochondrial pathway.

Treatment for ALPS patients is challenging. We have demonstrated rapamycin is potentially an effective medication against lymphoproliferation in ALPS and studies in humans have demonstrated it is well tolerated. Rapamycin causes little nephrotoxicity and neurotoxicity, unlike the immunophilins, but may cause hyperlipidemia and mild myelosuppression. We found no overt toxicities in mice treated with rapamycin for 3 months, although this study was designed as an efficacy study and not a long term murine toxicity study.

Other immunosuppressive agents have been studied in ALPS patients, including cyclosporine (CSA), mycophenolate mofetil (MMF) and corticosteroids. We compared rapamycin to MMF and found it to be superior in the mice. CSA is a calcineurin
inhibitor and decreases proliferation of T lymphocytes. CSA has been shown to have limited efficacy when used alone in ALPS but maybe useful in combination with corticosteroids.\textsuperscript{29,30} MMF inactivates inosine monophosphate, a key enzyme in purine synthesis, resulting in inhibition of proliferating T and B lymphocytes.\textsuperscript{10,31} MMF was recently demonstrated to be effective in reducing symptoms in a cohort of patients with ALPS, however, MMF is myelosuppressive inducing neutropenia as high as 20% of treated patients.\textsuperscript{32} MMF has also been associated with an increased risk of development of secondary malignancies.\textsuperscript{33} Patients with ALPS frequently have cytopenias and are already at risk for secondary malignancies, making these potential side effects of MMF worrisome. Corticosteroids induce apoptosis in B and T lymphocytes and are very efficacious in patients with ALPS;\textsuperscript{3,34,35} however, long term treatment with corticosteroids can be extremely toxic. Nevertheless, some patients with ALPS have less severe disease and mild immunosuppressive agents, including MMF, may be effective and safe.

Approximately 10% of patients with ALPS develop secondary malignancies, most commonly leukemia and lymphoma.\textsuperscript{7} Chronic exposure to immunosuppressive agents bears a theoretical risk for development of secondary malignancies, since the immune system is critical for tumor surveillance. Patients taking immunosuppressive agents, including MMF\textsuperscript{7}, after solid organ transplantation have been shown to have an increased risk of developing secondary lymphoma. Thus, it is important to use immunosuppressive agents with the least potential to induce malignant transformation in patients with ALPS. \textit{In vitro} data evaluating immunosuppressive drugs for mutagenic properties in human lymphocyte cultures demonstrated that tacrolimus and MMF are strongly mutagenic even
at low doses, CSA is strongly mutagenic but only at high doses, and rapamycin is not mutagenic unless given at very high concentrations and then is only weakly mutagenic.\textsuperscript{36}

A significant percentage of patients with ALPS develop autoimmune manifestations and treatments are frequently aimed at alleviating symptoms arising as a result of these autoimmune phenomenon. The murine model of ALPS we used for most of these studies, CBA-\textit{lpr}^{\textit{crg}}, typically develops lymphoproliferation and elevated DNTs, characteristic of ALPS; however, these mice do not develop significant autoimmune manifestations. Studies in children with ALPS have demonstrated a correlation between a decrease in DNTs and lymphoproliferation with improvement in autoimmune manifestations.\textsuperscript{10,37} MRL-\textit{lpr} mice are also phenotypically and genotypically similar to humans with ALPS. These mice develop more significant autoantibodies. They also develop glomerulonephritis and vasculitis, characteristics more similar with human systemic lupus erythematosus than ALPS.\textsuperscript{38} We, therefore, performed the majority of our experiments in the CBA-\textit{lpr}^{\textit{crg}} mice, and we used the MRL-\textit{lpr} mice to assess rapamycin’s effect on autoimmunity in the mice. We found rapamycin did reduce autoantibody burden in the MRL-\textit{lpr} mice. We are cautiously optimistic that the dramatic responses we have demonstrated in the mice may correlate to a reduction in autoimmune manifestations in patients with ALPS.

In summary, we have demonstrated that rapamycin is an effective agent in reducing DNTs, lymphadenopathy, splenomegaly, and autoantibodies in murine ALPS. Based on the results of these data, we plan to initiate a pilot study of rapamycin in children with ALPS that will hopefully lead to a safe, effective, and well-tolerated therapy for this difficult to treat disease.
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Rapamycin improves lymphoproliferative disease in murine autoimmune lymphoproliferative syndrome (ALPS)

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