

ARTICLE

Leniolisib reduced lymphoproliferative disease in murine autoimmune lymphoproliferative syndrome

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Autoimmune lymphoproliferative syndrome (ALPS) is an inborn error of immunity (IEI) characterized by abnormal FAS-mediated apoptosis of lymphocytes that leads to lymphoproliferation and expansion of CD4⁻/CD8⁻ double-negative T cells (DNTs). In patients with ALPS-FAS, DNTs have been reported to exhibit increased activity in the phosphoinositide 3-kinase δ (PI3Kδ)/mammalian target of rapamycin (mTOR) pathway. Although mTOR inhibition with sirolimus has improved autoimmune cytopenias and organomegaly in patients with ALPS, it requires monitoring of serum levels, and common adverse events frequently hamper long-term use. As leniolisib, a selective PI3Kδ inhibitor, reduced lymphoproliferation in another IEI known as activated PI3Kδ syndrome, efficacy was examined in a murine model of ALPS. Changes in organ weight and key immune subsets in MRL/lpr^{-/-} mice receiving vehicle or leniolisib (40 or 80 mg/kg/day) by oral gavage were assessed. Leniolisib limited the canonical features of ALPS, including lymphadenopathy, splenomegaly, and elevated DNTs, in a dose-dependent manner. These results support the evaluation of leniolisib in patients with ALPS (NCT06549114).

Introduction

Autoimmune lymphoproliferative syndrome (ALPS) is an inborn error of immunity (IEI) commonly caused by variants in FAS, the gene encoding the cell surface death receptor Fas. Germline or somatic variants in FAS can lead to apoptotic defects in lymphocytes, resulting in a subtype of ALPS known as ALPS-FAS (1, 2, 3). While germline variants in FAS are the most common, additional subtypes of ALPS have been reported in patients that are linked to other genetic variants such as TNFSF6 and CASP10. Regardless of the subtype, this disorder usually presents in childhood with splenomegaly, nonmalignant lymphadenopathy, chronic cytopenias, and elevated CD4⁻/CD8⁻ double-negative T cells (DNTs) (4). Patients with ALPS-FAS also have an increased risk of developing B cell lymphomas (3).

In individuals with ALPS-FAS, DNTs have been reported to exhibit increased activity in the phosphoinositide 3-kinase (PI3K)/mammalian target of rapamycin (mTOR) pathway (5). Specifically, ex vivo studies demonstrated enhanced phosphorylation of Akt, mTOR, and S6 in DNTs from patients with ALPS compared with those from healthy controls. ALPS DNTs treated with the mTOR inhibitor sirolimus had reduced proliferation and increased apoptosis. As PI3Kδ is predominately expressed in lymphocytes, these results further support a role for PI3Kδ/mTOR in the development of aberrant DNTs. Collectively, the evidence supports that hyperactive PI3Kδ/mTOR signaling along with altered programmed cell death contributes to

lymphoproliferation and expansion of CD4⁻/CD8⁻ DNTs in patients with ALPS-FAS (3, 5).

Treating ALPS can be challenging, especially in patients with refractory cytopenias and splenomegaly, the latter of which often develops at a young age. Currently, most therapeutics are either symptomatic or can produce serious or potentially severe adverse events (4). Sirolimus reduced organomegaly and improved autoimmune cytopenias in patients with ALPS; however, this treatment requires monitoring of serum levels and may lead to the development of oral mucositis and hyperlipidemia, which can result in discontinuation of treatment (6). It is cumbersome for clinicians to monitor and maintain trough levels of sirolimus in various patient populations, particularly in children who cannot swallow pills and require liquid formulations. Thus, there is a need for a treatment option that does not require strict monitoring due to a narrow therapeutic index, is well tolerated, and targets the multifaceted clinical components of ALPS. PI3Kδ is a potential therapeutic target that can be selectively inhibited with leniolisib, which is approved by the US Food and Drug Administration for patients ≥12 years of age with an IEI known as activated PI3Kδ syndrome (APDS) (7, 8).

Here, we assessed leniolisib treatment in MRL/lpr^{-/-} mice with pathogenic variants in the *lpr* gene located on chromosome 19, which is equivalent to human FAS on chromosome 10 (9, 10, 11). With virtually no detectable levels of fas protein, these mice

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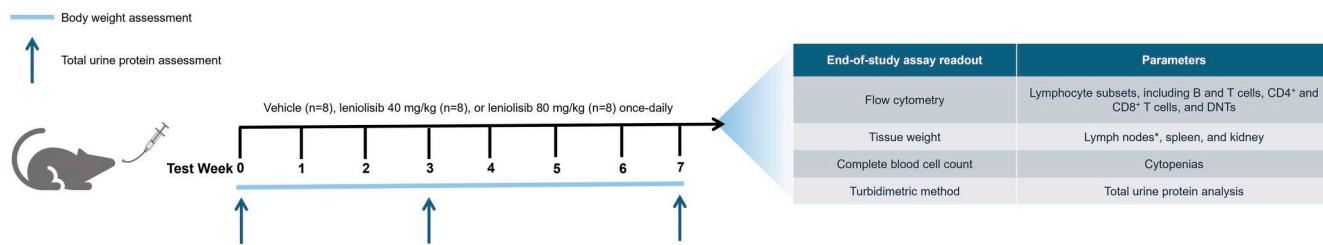


Figure 1. Experimental timeline and parameters assessed during treatment with leniolisib or vehicle. *Six lymph nodes were collected per mouse: two inguinal, two axillary, and two brachial.

develop an ALPS-like phenotype that includes autoimmunity, lymphadenopathy, splenomegaly, and expansion of CD4[−]/CD8[−] DNTs and have been used historically to evaluate the potential benefit of other therapeutic candidates (11, 12, 13, 14, 15, 16, 17, 18, 19, 20).

Results

The impact of leniolisib was assessed in a murine model of ALPS. Briefly, 6-wk-old female MRL/lpr^{−/−} mice received a daily dose of leniolisib (40 or 80 mg/kg) or vehicle (all groups, $n = 8$) by oral gavage for 7 wk (Fig. 1).

Clinical observations

No toxicities were observed in mice treated with leniolisib. Over the course of the study, mice did not develop any apparent infection or other clinical manifestations associated with immunosuppression. No statistically significant changes were noted in the percentage of live cells in the spleen, lymph nodes, blood, or bone marrow across all experimental groups. An increased number of palpable lymph nodes were observed in mice receiving vehicle compared with those receiving leniolisib. Nearly 17% of mice (three that received vehicle and one that received 80 mg/kg leniolisib) had skin lesions by the end of the study, supporting previous reports in the literature for this mouse model (21, 22). No significant differences between experimental groups in body weight changes from baseline weight were noted (Fig. 2 A). All dosing regimens were tolerated for the study duration.

Leniolisib limited lymphoproliferation

Compared with vehicle, mice treated with leniolisib had a significantly lower spleen weight. Specifically, the average spleen weight of mice administered 40 mg/kg leniolisib was 0.250 g vs. 0.348 g ($P = 0.0355$) in the vehicle group and 0.183 g for mice administered 80 mg/kg leniolisib ($P = 0.0005$) (Fig. 2 B). This difference was also observed when spleen weight was normalized to body weight, as mean spleen weight of mice administered 80 mg/kg leniolisib was 0.548% of their body weight vs. 0.875% in mice treated with vehicle ($P = 0.0469$) (data not shown). In the 80 mg/kg leniolisib group, the weight of lymph nodes was 0.118 g vs. 0.602 g in the vehicle group ($P < 0.0001$). When normalized to body weight, the weight of lymph nodes in the 80 mg/kg leniolisib group was also significantly lower than the vehicle group (0.348% of body weight vs. 1.549%; $P = 0.0027$) (data not

shown). Although not significant, the weight of lymph nodes was lower in mice treated with 40 mg/kg leniolisib compared with those administered vehicle (0.406 g vs. 0.602 g; $P = 0.0983$) (Fig. 2 C), which was also the case when normalized to body weight (1.234% of body weight vs. 1.549%; $P = 0.5835$) (data not shown). Overall, leniolisib limited both lymphadenopathy and splenomegaly in murine ALPS.

Leniolisib limited expansion of aberrant immune subsets

At the end of the study, we assessed the percentage of live cells, absolute counts, and frequency (as a percentage of CD3⁺ or CD45⁺ cells) of CD4[−]/CD8[−] DNTs and other immune subsets in the spleen, lymph nodes, blood, and bone marrow.

Overall, there was no change in the percentage of live cells out of total cells in the spleen, lymph nodes, blood, or bone marrow between all experimental groups (Fig. S1).

Spleen

In the spleen (Fig. 3 A), absolute counts of CD4[−]/CD8[−] DNTs were significantly lower in mice receiving 80 mg/kg leniolisib compared with mice administered vehicle (1.42×10^6 cells vs. 4.10×10^6 cells; $P < 0.0001$). Total CD3⁺ T cells were significantly lower in the 80 mg/kg leniolisib group compared with mice treated with vehicle (4.21×10^6 cells vs. 10.06×10^6 cells; $P < 0.0001$). Similarly, mice treated with 80 mg/kg leniolisib had significantly lower CD4⁺ T cells compared with those receiving vehicle (1.67×10^6 cells vs. 3.92×10^6 cells; $P = 0.0008$). In the B cell compartment, the 80 mg/kg leniolisib group had significantly lower absolute counts of CD19⁺ B cells compared with mice administered vehicle (3.15×10^6 cells vs. 6.61×10^6 ; $P < 0.0001$).

Unlike absolute counts of CD4⁺ T cells, there was no change in the percentage of CD4⁺ T cells out of CD3⁺ cells in the spleen (Fig. S2 A) between experimental groups. Although there was no change in absolute counts of CD8⁺ T cells between experimental groups, the percentage of CD8⁺ T cells out of CD3⁺ cells in the spleen was significantly higher in mice treated with 80 mg/kg leniolisib (26.79% vs. 19.33%; $P = 0.0007$) (Fig. S2 B). Finally, no significant changes were observed in the frequency of CD19⁺ B cells, CD3⁺ T cells, or CD4[−]/CD8[−] DNTs between spleens of experimental groups (data not shown).

Lymph nodes

In pooled analysis of lymph node samples (Fig. 3 B), which consisted of three lymph nodes from the right-hand side of each mouse (i.e., inguinal, brachial, and axillary), mice treated with

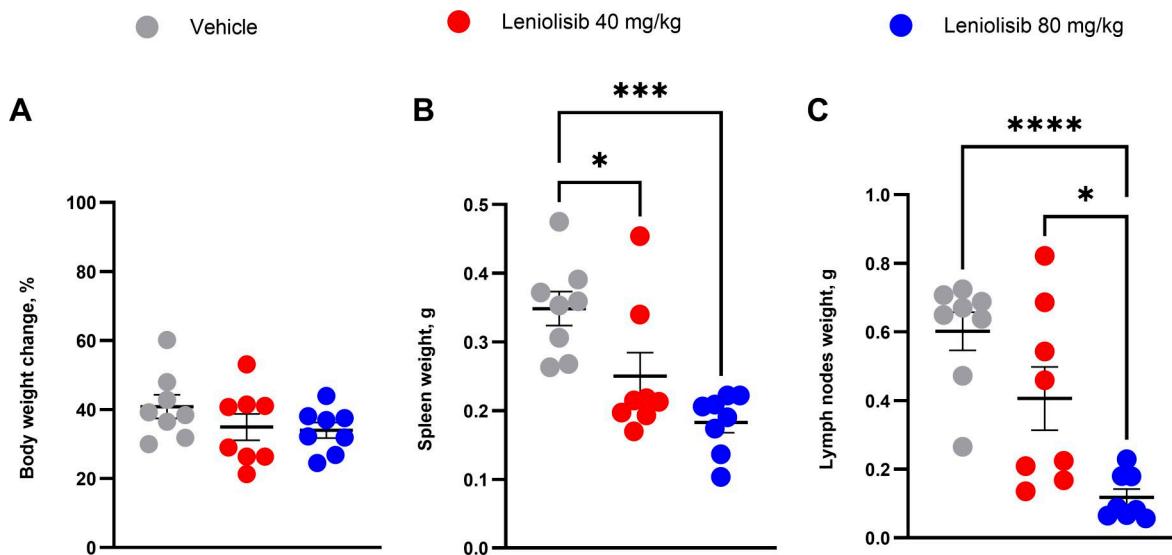


Figure 2. Body weight change and organ weight at the end of the study. (A–C) Individual and mean percent change in body weight from start weight (A). Individual and mean weight of spleen (B) and lymph nodes (C) following necropsy. Six lymph nodes (i.e., two inguinal, two axillary, and two brachial) from each mouse were used to determine weight (C). $n = 8$ per experimental group. Error bars are \pm SEM. Data were analyzed using a one-way ANOVA with Tukey correction. * $P < 0.05$; ** $P < 0.001$; *** $P < 0.0001$.

80 mg/kg leniolisib had significantly lower absolute counts of CD4[−]/CD8[−] DNTs compared with mice administered vehicle (1.96×10^6 cells vs. 17.43×10^6 cells; $P < 0.0001$). Total CD3⁺ T cells were also significantly lower in the 80 mg/kg leniolisib group (2.75×10^6 cells vs. 21.84×10^6 cells; $P < 0.0001$). No statistically significant changes in absolute counts of B cells, CD4⁺ T cells, or CD8⁺ T cells in lymph nodes were noted between experimental groups.

Similar to absolute counts in pooled lymph node samples, mice treated with 80 mg/kg leniolisib had a significantly lower percentage of CD4[−]/CD8[−] DNTs out of CD3⁺ T cells compared with mice receiving vehicle (52.39% vs. 78.48%; $P = 0.0009$) (Fig. S2 C). However, the percentages of CD4⁺ T cells, CD8⁺ T cells, and CD19⁺ B cells were significantly higher in mice administered leniolisib compared with mice administered vehicle. Specifically, the percentage of CD4⁺ T cells out of CD3⁺ T cells was significantly higher in mice administered 80 mg/kg leniolisib compared with those administered vehicle (22.53% vs. 12.39%; $P = 0.0034$) (Fig. S2 D). In the 80 mg/kg leniolisib group, the percentage of CD8⁺ T cells was higher compared with the vehicle group (24.94% vs. 9.07%; $P = 0.0009$) (Fig. S2 E). In the B cell compartment, mice treated with 80 mg/kg leniolisib had a significantly higher percentage of CD19⁺ B cells out of CD45⁺ cells compared with mice treated with vehicle (22.21% vs. 5.48%; $P = 0.0019$) (Fig. S2 F). No significant change was detected in the percentage of CD3⁺ T cells between experimental groups.

Blood

In terminal blood samples (Fig. 3 C), mice administered 80 mg/kg leniolisib had significantly lower absolute counts of total CD3⁺ T cells compared with mice receiving vehicle (80.32 cells/ μ L vs. 201.4 cells/ μ L; $P = 0.0020$). Although not significant when compared with mice administered vehicle, absolute CD4[−]/CD8[−] DNTs were numerically lower in mice treated with

80 mg/kg leniolisib (18.47 cells/ μ L vs. 99.81 cells/ μ L; $P = 0.0534$). No significant changes were noted in absolute counts of B cells, CD4⁺ T cells, or CD8⁺ T cells between experimental groups.

Although not significant for absolute counts, the percentage of CD4[−]/CD8[−] DNTs out of CD3⁺ T cells was significantly lower in blood of mice treated with 80 mg/kg leniolisib compared with mice receiving vehicle (26.09% vs. 46.91%; $P = 0.0119$) (Fig. S2 G). Compared with vehicle, no significant changes were observed in the percentage of CD3⁺ T cells, CD4⁺ T cells, CD8⁺ T cells, or CD19⁺ B cells between experimental groups.

Bone marrow

No significant changes in absolute cell counts (Fig. 3 D) or frequency of immune subsets (data not shown) were detected in the bone marrow between experimental groups.

Collectively, absolute counts of total CD3⁺ T cells as well as CD4[−]/CD8[−] DNTs, a biomarker of ALPS, in the spleen, lymph nodes, and blood were significantly lower in mice receiving 80 mg/kg leniolisib. In the spleen, mice administered 80 mg/kg also had significantly lower absolute counts of CD4⁺ T cells and CD19⁺ B cells.

Mice treated with leniolisib had lower absolute white blood cell counts, lymphocytes, and monocytes

At the end of the study, terminal blood samples were collected to determine the effect of leniolisib on blood cell counts.

Mice treated with 80 mg/kg leniolisib had significantly lower absolute counts of white blood cells, lymphocytes, and monocytes compared with mice administered vehicle (Fig. 4, A–C). Specifically, mean absolute counts of white blood cells in mice administered 80 mg/kg leniolisib were 1.75×10^9 /L vs. 3.90×10^9 /L in mice treated with vehicle ($P = 0.0106$), lymphocytes were 1.62×10^9 /L vs. 3.65×10^9 /L ($P = 0.0110$), and monocytes were 0.05×10^9 /L vs. 0.11×10^9 /L ($P = 0.0116$). Monocytes were

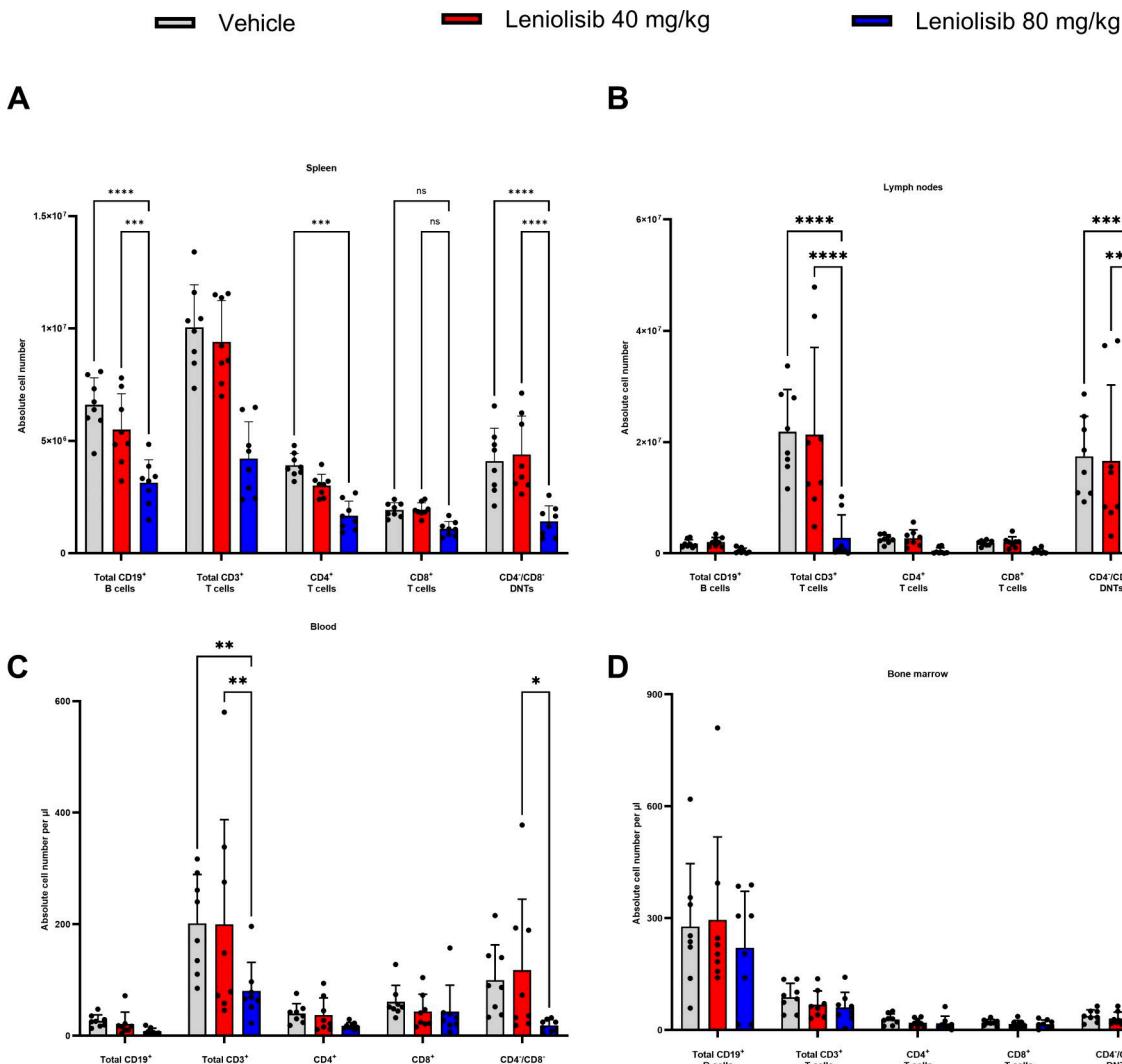


Figure 3. Changes in immune subsets at the end of the study. (A-D) Individual and mean absolute B and T cell counts in the spleen (A), lymph nodes (B), blood (C), and bone marrow (D). Half of the spleen (A) and three right-hand side lymph nodes (B) were processed for flow cytometry. $n = 8$ per experimental group. Error bars are \pm SEM. Data were analyzed using a two-way ANOVA with Tukey correction. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

also significantly lower in the 40 mg/kg group ($0.06 \times 10^9/L$; $P = 0.0397$). No significant changes were noted in neutrophils, eosinophils, or basophils (Fig. 4, D-F) between experimental groups.

Leniolisib increased several hematologic parameters

Hemoglobin and hematocrit levels, red blood cell (RBCs) counts, and red cell distribution width (RDW) were significantly higher in mice receiving leniolisib compared with mice administered vehicle, while mean platelet volume was significantly lower. Specifically, hemoglobin levels were 165.6 g/L in the 40 mg/kg leniolisib group ($P = 0.0071$) and 167.8 g/L in the 80 mg/kg group ($P = 0.0011$) vs. 155.5 g/L in mice receiving vehicle (Fig. 4 G). Hematocrit levels were 0.50 L/L ($P = 0.0100$) in mice treated with 40 mg/kg leniolisib and 0.49 L/L in mice treated with 80 mg/kg leniolisib ($P = 0.0180$) vs. 0.46 L/L in mice receiving vehicle. Absolute counts of RBCs were significantly higher in both leniolisib groups; RBCs were $9.41 \times 10^9/L$ ($P = 0.0137$) in

mice administered 40 mg/kg leniolisib and $9.63 \times 10^9/L$ in those administered 80 mg/kg leniolisib ($P = 0.0007$) vs. $8.87 \times 10^9/L$ in mice treated with vehicle (Fig. 4 H). In mice treated with 80 mg/kg leniolisib, RDW was significantly higher compared with mice receiving vehicle (18.23% vs. 16.66%; $P = 0.0007$; data not shown). Mean platelet volume was significantly lower in mice treated with 40 mg/kg leniolisib compared with those treated with vehicle (5.41 fL vs. 5.94 fL; $P = 0.0050$; data not shown), but no changes were observed in the number of platelets (Fig. 4 I). Finally, no significant changes were observed in mean cell levels of hemoglobin, concentration of hemoglobin, or RBC volume compared with vehicle (data not shown).

Leniolisib reduced total urine protein

In addition to outcomes related to ALPS, total urine protein was assessed at baseline and at 21 and 49 days of treatment as a proxy to determine the impact of leniolisib on renal function. MRL/lpr^{-/-} mice often experience progressive renal failure and have

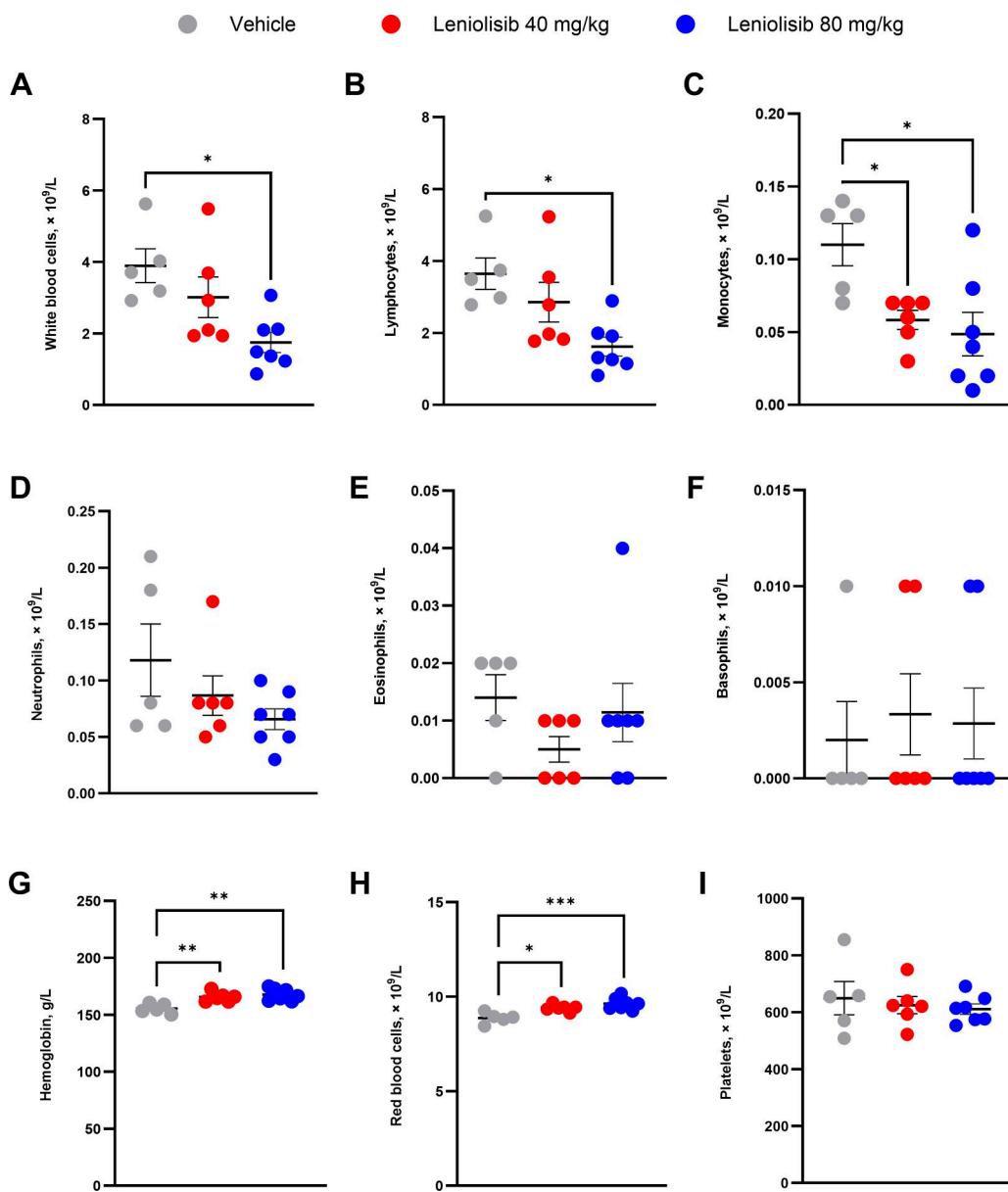


Figure 4. CBCs and hematologic readouts of terminal blood samples at the end of the study. (A-I) Individual and mean absolute white blood cell (A), lymphocyte (B), monocyte (C), neutrophil (D), eosinophil (E), and basophil (F) counts. Individual and mean hemoglobin (G), RBCs (H), and platelets (I). *n* values for vehicle, 40 mg/kg leniolisib, and 80 mg/kg leniolisib are 5, 6, and 7, respectively. Error bars are \pm SEM. Data were analyzed using a one-way ANOVA with Tukey correction. *P < 0.05; **P < 0.01; ***P < 0.001.

been utilized as a model of lupus nephritis (11, 23, 24, 25). Leniolisib treatment groups showed a stabilization or reduction in total urine protein, while the vehicle group experienced an expected increase due to development of the disease (Fig. 5, A and B) (23). Specifically, the percent change in total protein from baseline decreased in mice receiving 40 and 80 mg/kg leniolisib but increased in mice receiving vehicle. At day 21, 40 mg/kg leniolisib decreased total urine protein to 47.0% of the baseline value, and 80 mg/kg decreased it to 70.2% of the baseline value vs. an increase to 166.7% of the baseline value with vehicle. At day 49, total urine protein decreased to 64.9% of the baseline value, and 80 mg/kg decreased it to 97.1% of the baseline value with an increase to 150% of the baseline value with

vehicle. No significant differences between experimental groups were observed in kidney weight (Fig. 5 C) nor when kidney weight was normalized to body weight.

Discussion

Overall, leniolisib induced a dose-related reduction in canonical clinical features of ALPS-FAS in the murine model, including lymphadenopathy, splenomegaly, and elevated CD4⁺/CD8⁺ DNTs in blood, spleen, and lymph nodes, and thus reduced the overall disease burden.

Aberrant DNTs of patients with ALPS-FAS have been reported to exhibit increased activity of the PI3K/mTOR-

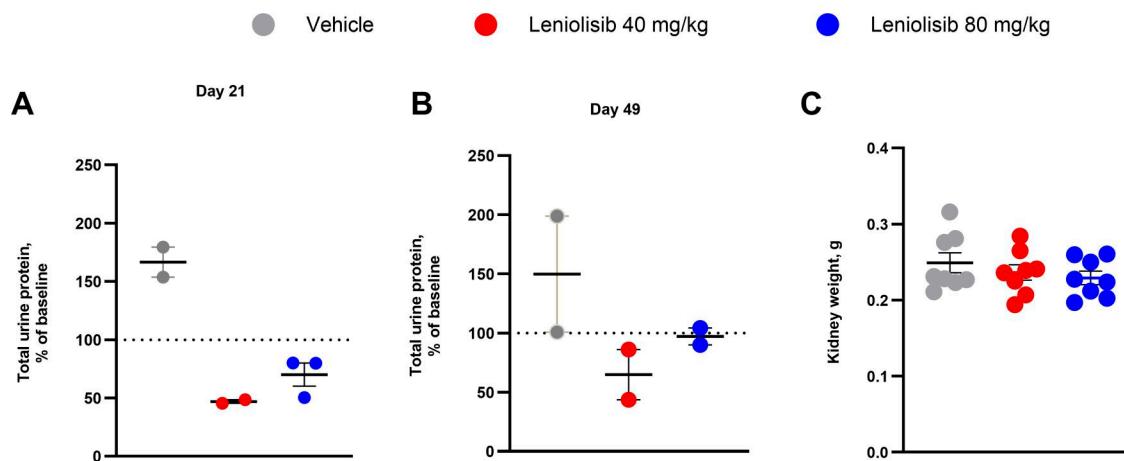


Figure 5. Total urine protein in pooled urine samples over time and kidney weight at the end of the study. (A–C) Percent change in total urine protein from baseline values was assessed at day 21 (A) and day 49 (B) of the study. Individual and mean kidney weight (C) at the end of the study. As a minimum of 150 μ L of urine was required for total protein analysis, urine samples were pooled in A and B. Pooled samples were extracted from one to four mice per experimental group, and protein analysis was completed in two to three replicates. One kidney (right side) from each mouse was used to determine weight (C). Error bars are \pm SEM. Data in C were analyzed using a one-way ANOVA with Tukey correction ($P < 0.05$).

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dependent pathway (5). Therefore, we hypothesized that inhibition of PI3K δ with leniolisib would improve lymphoproliferation via a reduction in elevated DNTs and other accumulated lymphocyte populations. In this study, the weights of the spleen and lymph nodes along with absolute counts of CD4 $^+$ /CD8 $^+$ DNTs and CD3 $^+$ T cells were significantly lower in mice treated with leniolisib when compared with vehicle. In terminal blood samples, absolute counts of CD3 $^+$ T cells were significantly lower with leniolisib than with vehicle. Compared with vehicle, absolute counts of white blood cells, lymphocytes, and monocytes were significantly lower in mice treated with leniolisib, while RBCs, RDW, and hemoglobin and hematocrit levels were higher. Elevated urine protein levels, which are commonly reported in this mouse model and serve as an indicator of autoimmune glomerulonephritis, were absent in mice receiving leniolisib (23). Importantly, no overt toxicities were reported in mice treated with up to 80 mg/kg/day of leniolisib for 7 wk via oral gavage. Overall, we demonstrated that leniolisib limited disease burden in a murine model of ALPS.

The results from the current preclinical work have significant implications within the clinical domain. Nearly all patients with ALPS-FAS develop lymphadenopathy and splenomegaly and are at an increased risk for developing lymphoma (3). Lymphoproliferation in these patients has been attributed to the accumulation of pathognomonic CD4 $^+$ /CD8 $^+$ DNTs (3, 26). Seminal studies illustrated that splenectomy can effectively reduce lymphoproliferation in MRL/lpr $^{-/-}$ mice; however, post-splenectomy sepsis is a major cause of morbidity and mortality among patients with ALPS (3, 27). Therefore, we sought to investigate an alternative immunomodulatory approach that could address lymphoproliferation. Oral administration of leniolisib decreased total counts of lymphocytes and DNTs as well as spleen weight in MRL/lpr $^{-/-}$ mice, further suggesting that a reduction in DNTs may be associated with a proportional decrease in lymphoproliferation in ALPS, but future studies are needed to gain a comprehensive

understanding of the underlying mechanism (4, 5, 28, 29, 30).

Another prominent manifestation in patients with ALPS is recurrent multilineage cytopenias, which are reported in \sim 70% of patients and often require multiple agents for treatment (3). Although the mouse model used in the current study lacks a specific cytopenia phenotype, an increase in hemoglobin and hematocrit levels, absolute counts of RBCs, and RDW support the notion that leniolisib may improve cytopenias for patients with ALPS. Moreover, treatment with the selective PI3K δ inhibitor parsacalisib improved hemoglobin levels in adults with autoimmune hemolytic anemia, further supporting PI3K δ inhibition as a potential therapeutic approach for autoimmune cytopenias, which are common and can be refractory in patients with ALPS (4, 31). Given that increased PI3K signaling in lymphocytes has been reported in patients with various autoimmune conditions, we speculate that PI3K δ inhibitors may improve autoimmune cytopenias by reducing elevated levels of autoantibodies (32). Historically, sirolimus significantly reduced autoantibodies in MRL/lpr $^{-/-}$ mice, further supporting PI3K/mTOR inhibition to improve autoimmunity (30). However, further studies are needed to confirm this.

While nephritis has only been reported in a limited subset of patients with ALPS, it is a known phenotype in MRL/lpr $^{-/-}$ mice (23, 33, 34). As these mice often experience glomerulonephritis, they are commonly used as a preclinical model of lupus nephritis (35, 36, 37). Consistent with findings from the current preclinical study, leniolisib improved proteinuria in a patient with APDS who experienced refractory lupus nephritis (38). These results support the notion that leniolisib may improve renal manifestations associated with ALPS, such as nephritis, but further studies are needed.

Currently, the treatment landscape for ALPS can be challenging for clinicians to navigate, with many options addressing only specific manifestations or inducing adverse events. For example, most patients with ALPS require treatment at a very

young age for refractory autoimmune cytopenias (3). First-line treatment for ALPS often includes corticosteroids, which may lead to serious adverse events, such as hypertension, bone fractures, and avascular necrosis, with long-term use (39, 40). The most common immunomodulatory drugs used for the treatment of ALPS are mycophenolate mofetil (MMF) and sirolimus. In clinical trials, treatment with MMF improved autoimmune cytopenias in patients with ALPS while inducing hypogammaglobulinemia in some; however, MMF had little to no effect on lymphoproliferation or elevated CD4⁺/CD8⁺ DNTs (40, 41). Similarly, sirolimus improved autoimmune cytopenias and organomegaly in patients with ALPS but requires long-term monitoring of serum levels and can induce adverse events such as mucositis, hypertension, and hypertriglyceridemia (6). Therefore, well-tolerated and effective therapeutics are needed to manage manifestations associated with ALPS.

In mice, several treatment approaches targeting autoimmune features have been studied, including immunosuppressive compounds that largely target T cells, antirheumatics, anti-parasitics, and surgical procedures such as thymectomy, splenectomy, and allogeneic bone marrow transplantation (13, 15, 17, 18, 42, 43, 44, 45, 46, 47, 48). While these approaches have shed light on the potential mechanisms of autoimmunity in ALPS, some failed to ameliorate manifestations, and many relied on immunosuppressive agents that may not be ideal for long-term use (e.g., valproic acid, pyrimethamine, and MMF) (13, 15, 17). The impact of sirolimus was assessed in both MRL/lpr^{-/-} and CBA-lpr^{cg} mice (30, 49). In the latter, mice administered sirolimus experienced a reduction in lymphoproliferation and CD4⁺/CD8⁺ DNTs (30). In MRL/lpr^{-/-} mice, sirolimus reduced lymphoproliferation and urine protein levels, but its effect on CD4⁺/CD8⁺ DNTs was not reported (49). In our current work, we illustrated leniolisib can not only improve elevated urine protein levels and limit lymphoproliferation in MRL/lpr^{-/-} mice but can also limit the expansion of CD4⁺/CD8⁺ DNTs.

Considering leniolisib does not require strict monitoring of serum levels and was well tolerated in patients ≥ 12 years of age with APDS, we are optimistic that this selective PI3K δ inhibitor could prove to be an effective treatment for patients, especially in children with ALPS who experience significant lymphoproliferation (8, 50). Currently, a clinical trial is recruiting patients with either germline or somatic variants in FAS (NCT06549114). The trial aims to assess the safety of leniolisib and examine clinical measures such as cytopenias and lymphoproliferation. Additionally, two pediatric trials are currently assessing the safety and efficacy of leniolisib in pediatric patients with APDS between the ages of 1–6 and 4–11 years (NCT05693129 and NCT05438407, respectively). Notably, leniolisib will be administered to patients 1–6 years of age as granules, which is suitable for children who cannot swallow pills.

This study has several limitations. Despite the potential to limit generalizability but consistent with other studies assessing ALPS-FAS, only female MRL/lpr^{-/-} mice were used in the current proof-of-concept study, as disease manifestations are accelerated and more severe compared with males (41, 49, 51). Additionally, several mice across different experimental groups (6 of 24) were excluded from complete blood cell count (CBC)

analysis (see Materials and methods), reducing the sample size for this outcome measure. Similarly, urine protein analyses required samples be pooled from mice, reducing the overall sample size per experimental group for this measure. As patients with ALPS often experience defects in apoptosis, the impact of leniolisib on these mechanisms as well as on levels of interleukin 10, soluble Fas ligand, lipids, vitamin B12, and phosphorylated AKT/S6 levels warrants further investigation. Additionally, as MRL/lpr^{-/-} mice experience autoreactivity that is suspected to accelerate the development of lymphoproliferation and autoimmunity, assessment of autoantibodies in mice receiving leniolisib is another potential area of study (52). These and additional future studies may further build upon the evidence presented here, which suggests that leniolisib may be an effective agent for treatment of children and adults with ALPS.

Materials and methods

Animals and leniolisib treatment schedule

Experimental support was provided by Syngniture Discovery. This work was approved by the Institutional Animal Welfare and Ethical Review Body and carried out under the United Kingdom Home Office Project License in accordance with the Animals Scientific Procedures Act of 1986 and the European Directive 2010/63/EU. A sample size of 7 animals per group was deemed suitable based on a statistical power of 95% and an alpha value of 0.05. An additional animal was included per group for a total of 8 mice per experimental group to mitigate risk of data loss due to disease-related mortality. All experiments in this in vivo study with 8 mice per treatment group were conducted once. 6-wk-old female MRL/MpJ-Fas^{lpr}/J (MRL/lpr^{-/-}) mice (strain # 000485; Jackson Laboratories) received a daily dose of leniolisib (40 or 80 mg/kg) or vehicle via oral gavage for 7 wk (all groups, $n = 8$). Vehicle consisted of 0.5% (weight per weight) methylcellulose and 0.5% (weight per weight) Tween 80 (Polysorbate 80) aqueous solution.

Clinical observations and sample collection

All animals had a full health check once daily that included palpation of lymph nodes to check for lymphadenopathy. Body weight was determined daily for the duration of the study. Urine samples were collected at day -1 (baseline) and at days 21 and 49 post treatment and pooled within experimental groups (a minimum of 150 μ l was required for protein analysis). Pooled samples were extracted from one to four mice per experimental group, and urine analysis was completed in two to three replicates.

24 h after the final dose, mice were euthanized, and terminal blood, bone marrow, spleens, lymph nodes, and kidneys were collected. Terminal blood samples were collected from all animals. Part of the sample was used for CBC analysis, while the other was used for flow cytometry. Both femurs and half of the spleen from each mouse were collected and stored in phosphate-buffered saline (lot #20012027; Gibco) on ice prior to analysis. Six lymph nodes (two inguinal, two axillary, and two brachial) from each mouse were weighed, while three from each mouse were combined for flow cytometry analysis. One kidney from each mouse was weighed.

Flow cytometry analysis

Study samples were processed and stained for flow cytometry analysis immediately after necropsy.

Blood, spleen, lymph nodes, and bone marrow cellularity were assessed by isolating single-cell suspensions and counting live cells using live/dead fixable viability dye (diluted 1:1,000, catalog #65-0866-14; eBioscience). Lymphocyte subsets were assessed via flow cytometry of single-cell suspensions using the following antibodies: CD45 (diluted 1:500, clone 30-F11, catalog #63-0451-82; eBioscience), CD4 (diluted 1:500, clone RM4-5, catalog #100557; BioLegend), CD8 (diluted 1:100, clone 53-6.7, catalog #100706; BioLegend), CD19 (diluted 1:1,000, clone 1D3, catalog #152405; BioLegend), and CD3 (diluted 1:40, clone 17A2, catalog #56-0032-82; eBioscience). Markers used to identify lymphocyte subsets were as follows: B cells, CD45⁺CD19⁺; CD4⁺ T cells, CD3⁺CD4⁺CD8⁻; CD8⁺ T cells, CD3⁺CD8⁺CD4⁻; DNTs, CD3⁺CD4⁻CD8⁻. Total CD3⁺ T cells included CD4⁺, CD8⁺, and DNTs. Samples were examined using an Attune NxT flow cytometer (Thermo Fisher Scientific) within 24 h of staining. Representative sample gating for the spleen can be found in Fig. S3. Compensation matrices for each panel were determined using UltraComp eBeads (lot #01-2222-41; Thermo Fisher Scientific). Flow cytometry standard files (.fcs) were analyzed using FlowJo v10.8.1 software.

Total urine protein analysis

Total urine protein expressed as a percentage of baseline at the midpoint (21 days) and end-of-study (49 days) was determined using the turbidimetric method. Absorbance was measured at 505 nm. Urine samples were analyzed within a week of collection.

CBC analysis

Terminal blood samples were collected and analyzed within 24 h of collection using the Coulter Principle (53). A DxH 520 Hematology Analyzer (lot #B40602; Beckman Coulter) was used to count the individual cells and provide cell size distribution. Collectively, 6 of 24 mice were excluded (leniolisib, $n = 3$; vehicle, $n = 3$) from CBC analysis due to insufficient blood volume ($n = 3$) or visible clots ($n = 3$).

Data analysis

All graphs were plotted using GraphPad Prism v10.2.3. Statistical analyses were performed using either a one- or two-way ANOVA with Tukey correction for multiple comparisons. A P value < 0.05 with 95% confidence was considered significant.

Online supplemental material

Fig. S1 illustrates the frequency of live cells at the end of the study in the spleen, lymph nodes, blood, and bone marrow. Fig. S2 details changes in the frequency of select immune subsets at the end of the study in the spleen, lymph nodes, and blood. Fig. S3 shows representative flow cytometry sample gating for the spleen.

Data availability

The data are available upon reasonable request. Researchers who have a methodologically sound research proposal should

send inquiries or requests to info@pharming.com. Data requestors may be required to sign a data access agreement.

Acknowledgments

The authors thank Signature Discovery for experimental support and Jaki DeFinis, PhD, Carolyn Keating, PhD, and the staff at Precision AQ for assistance with manuscript preparation.

Author contributions: Chopie Hassan: conceptualization, data curation, formal analysis, investigation, methodology, project administration, resources, validation, visualization, and writing—original draft, review, and editing. Yolanda Ponstein: conceptualization, methodology, and writing—review and editing. Robert Hanssen: conceptualization, funding acquisition, methodology, resources, supervision, visualization, and writing—review and editing. Kevin S. Thorneloe: conceptualization, funding acquisition, project administration, supervision, and writing—review and editing. Rebecca A. Marsh: methodology and writing—review and editing. V. Koneti Rao: conceptualization, methodology, and writing—original draft, review, and editing.

Disclosures: K.S. Thorneloe reported “other” from Pharming outside the submitted work; and being an employee and shareholder of Pharming. R.A. Marsh reported “other” from Pharming Healthcare outside the submitted work; and is employed part-time by Pharming Healthcare, Warren, NJ. V.K. Rao reported “other” from Pharming, Inc (CRADA) and Corvus Pharma (CRADA) during the conduct of the study. R. Hanssen, C. Hassan and, Y. Ponstein are employees of Pharming. No other disclosures were reported.

Submitted: 25 June 2025

Revised: 5 September 2025

Accepted: 28 October 2025

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Supplemental material

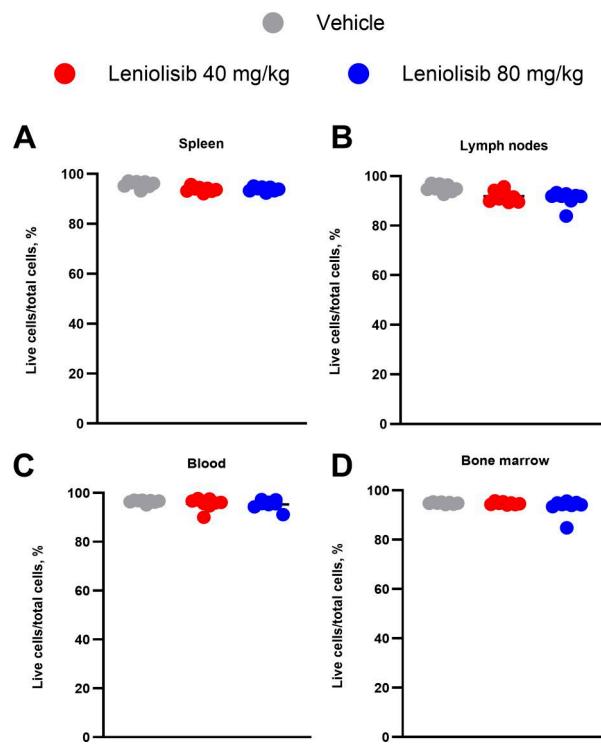


Figure S1. Frequency of live cells at the end of the study. (A-D) Frequency of live cells in the spleen (A), lymph nodes (B), blood (C), and bone marrow (D). Half of the spleen and three lymph nodes from the right side of each mouse were used for detection of live cells in A and B, respectively. $n = 8$ per experimental group. Error bars are \pm SEM. Data were analyzed using a one-way ANOVA with Tukey correction ($P < 0.05$).

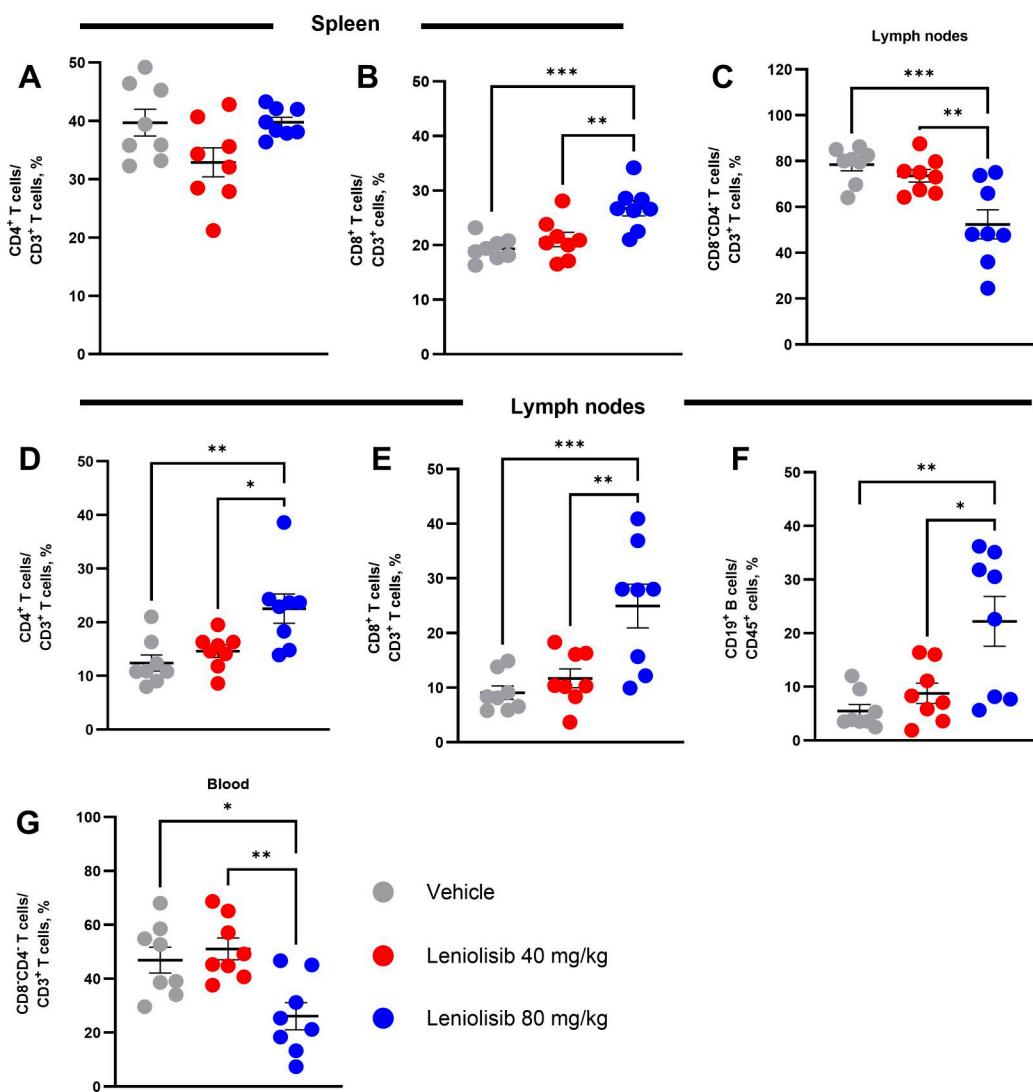


Figure S2. Changes in frequency of select immune subsets at the end of the study. (A–G) Individual and mean CD4⁺ (A) and CD8⁺ (B) T cell frequency in the spleen. Individual and mean CD8[−]/CD4[−] DNT (C), CD4⁺ T cell (D), CD8⁺ T cell (E), and CD19⁺ B cell (F) frequencies in the lymph nodes. Individual and mean CD8⁺ T cell frequency in the blood (G). $n = 8$ per experimental group. Error bars are \pm SEM. Data were analyzed using a one-way ANOVA with Tukey correction. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

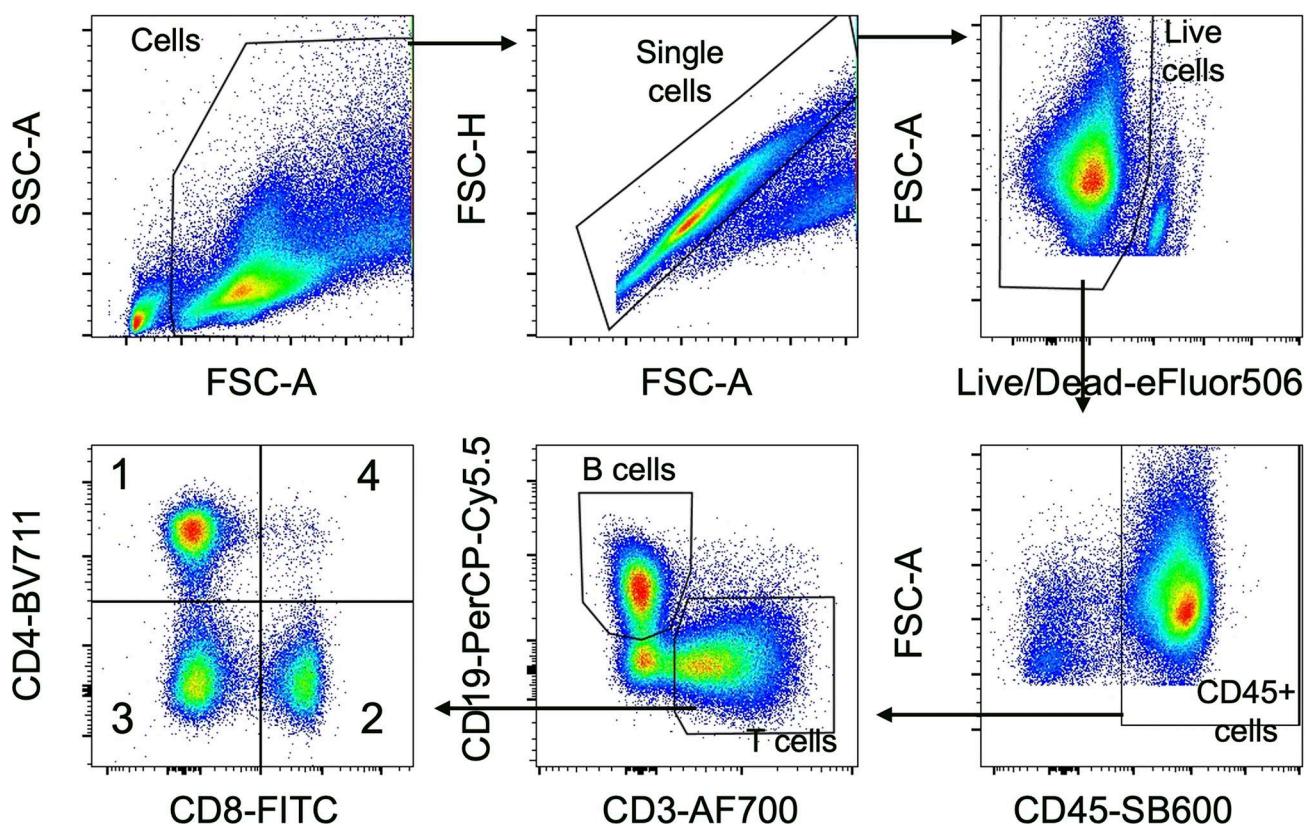


Figure S3. Representative flow cytometry sample gating for the spleen. AF, Alexa Fluor; BV, Brilliant Violet; FITC, fluorescein isothiocyanate; FSC-A, forward scatter area; FSC-H, forward scatter height; PerCP-Cy5.5, peridinin chlorophyll protein Cy5.5; SB, SuperBright; SSC-A, side scatter area.