PI3K/AKT/mTOR hypersignaling in autoimmune lymphoproliferative disease engendered by the epistatic interplay of Sle1b and FAS\textsuperscript{lp}\textsuperscript{r}

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Abstract

Previous studies have demonstrated that the NZM2410/NZW ‘z’ allele of Sle1 on telomeric murine chromosome 1 led to lymphoproliferative autoimmunity, when acting in concert with the FAS\textsuperscript{lp}\textsuperscript{r} defect on the C57BL/6 background. The present report shows that the Sle1b sub-locus, harboring the NZM2410/NZW ‘z’ allele of SLAM, in epistasis with FAS\textsuperscript{lp}\textsuperscript{r}, may be sufficient to induce lymphoproliferative autoimmunity. Disease in this simplified genetic model is accompanied by significant activation of the AKT signaling axis in both B- and T cells, as evidenced by increased phosphorylation of AKT, mTOR, 4EBP-1 and p70S6K, resulting from increased PI3K and reduced PTEN activity. In addition, blocking this axis using RAD001, an mTOR inhibitor, ameliorated lymphoproliferation and modulated serum IgG anti-nuclear auto-antibodies. Finally, mTOR inhibition also dampened signaling via parallel axes, including the MAPK and NFkB pathways. Hence, hypersignaling via the PI3K/AKT/mTOR axis appears to be an important mechanism underlying autoimmune lymphoproliferative disease, presenting itself as a potential target for therapeutic intervention.

Introduction

Autoimmune lymphoproliferative syndrome (ALPS) is a disease marked by autoimmunity and lymphoproliferation, resulting as a consequence of impaired lymphocytic apoptosis (1–7). Mutations in FAS/FASL and caspases have been associated with ALPS. Nevertheless, it has been recognized that mutations in these genes alone may not be sufficient to engender ALPS (8, 9). For instance, it is well documented that relatives of ALPS patients may harbor the same culprit mutations as the proband, but may not have any phenotypic manifestations. This alludes to the potential contribution of additional genetic factors in disease pathogenesis. Indeed, the above scenario has been faithfully reproduced in animal models. Whereas mice with the FAS\textsuperscript{lp}\textsuperscript{r} mutation exhibit rather modest humoral and cellular phenotypes, epistatic interaction with the NZM2410/NZW ‘z’ allele of the lupus susceptibility locus, Sle1, leads to full-blown lymphoproliferative lupus, characterized by anti-nuclear antibodies, splenomegaly, lymphadenopathy, nephritis and early mortality (10).

Intriguingly, mice with PTEN (phosphatase and tensin homolog) haploinsufficiency or PI3K overactivity, both develop ALPS that is phenotypically similar to the disease seen in B6.Sle1.- FAS\textsuperscript{lp}\textsuperscript{r} mice (11, 12). Importantly, PTEN and PI3K operate in diametrically opposite fashions, to regulate the cellular content of activated AKT (13–16). As one might have predicted, mice that hyperexpress AKT in their lymphocytes also succumb to ALPS (17). It has also become apparent that overactivity of this pathway in B cells as well as T cells may lead to ALPS (17–19). In view of these observations, an important goal of this study was to ascertain if the PI3K/AKT/mTOR axis was hyperactivated in B6.Sle1.FAS\textsuperscript{lp}\textsuperscript{r} mice. Since this axis has been proven to be an excellent target for therapeutic intervention in other disease states (20, 21), a further goal of this study was to ascertain if lymphoproliferative lupus in B6.Sle1.FAS\textsuperscript{lp}\textsuperscript{r} mice could also be mitigated by targeting this axis.

The NZM2410/NZW Sle1 interval by itself leads to low-grade autoimmunity (22, 23). It has become clear that the

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Sle1 lupus susceptibility interval on mouse chromosome 1 is composed of three sub-loci, Sle1a, Sle1b and Sle1c (24). Among these, Sle1b is the strongest locus being associated with the highest levels or penetrance of auto-antibodies (24). Recently, it has been shown that polymorphic variants of the SLAM family of molecules constitute the culprit genes for Sle1b (25). Hence, to accomplish the aforementioned goals, the present study utilizes a newly generated B6.Sle1bNZM2410/FASpo strain (referred to as B6.Sle1b/lpr in this manuscript) that is double homozygous for FASpo and the NZM2410/NZW allele of Sle1b on chromosome 1.

Methods

Mice

C57BL/6 (B6) mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and subsequently bred in our animal colony. B6.Sle1bNZM2410/NZW mice, simply referred to as B6.Sle1b+ (or B6.Sle1b), are B6 mice rendered congenic for the NZM2410-derived Sle1b on chromosome 1 with termini at D1mit113 and D1mit407, excluding Sle1a and Sle1c. B6.FASpo (or B6.lpr) mice are B6 mice bearing the lpr mutation of FAS on a B6 background, exhibiting modest autoimmunity (26), obtained from the Jackson Laboratory. B6.Sle1b+/lpr mice expressing Sle1b+ and FASpo+, both homozygously, were derived by breeding B6.Sle1b+ with B6.lpr, and then crossing the double heterozygous offspring with each other and selecting progeny that were homozygous at both loci. All mice used for this study were bred and housed in a specific pathogen-free colony at UT Southwestern Medical Center, Department of Animal Resources, in Dallas, TX, USA. Both male and female mice were used, and any observed sex differences are noted.

Flow cytometric analysis and antibodies

Splenocyte preparations were depleted of red blood cells using tris–ammonium chloride, and single cell suspensions were prepared for flow cytometric analysis. Lymph nodes (LNs) were obtained from the inguinal sites, and crushed to obtain single cell suspensions. Peritoneal cavity cells were obtained per sample. The mean linear units on the for- or not they expressed surface CD5. In addition, the percentages of follicular B cells (B220+ve, CD23+ve, CD21lo), and marginal zone B cells (B220+ve, CD23hi, CD21hi) were also defined. B220+ve, CD21lo, CD23lo B cells were identified as being pre-plasmablasts predominantly based on their surface expression of sydsecan-1, CD43 and other markers as indicated.

T-cell functional studies

For the functional studies, splenic CD4+ T cells were purified from the indicated strains using anti-CD4 magnetic beads (Miltenyi Biotec, Auburn, CA, USA) and were >95% pure. For the T-cell proliferation assays, the purified splenic T cells were stimulated with 1 μg ml⁻¹ plate-bound anti-CD3 for 24 or 96 h. The degree of cell division was gauged from the serial diminution of CFSE (carboxy-fluorescein diacetate, succinimidyl ester), as determined by flow cytometry. For gauging the degree of apoptosis, the cells were stained with Annexin V (PharMingen) and 7-AAD (7-amino-actinomycin D, Calbiochem) and examined by flow cytometry at the indicated time points.

ELISA for auto-antibodies

The anti-dsDNA, anti-ssDNA, anti-histone and anti-histone/DNA ELISA assays were carried out as described before (10). Raw OD (optical density) was converted to U ml⁻¹ using a positive control serum derived from a B6.Sle1/lpr mouse, arbitrarily setting the reactivity of a 1:100 dilution of this serum to 100 U/ml. Test sera with reactivity stronger than the standard were diluted further and re-assayed. The glomerular-binding ELISA was performed as described previously (10) using sonicated rat glomeruli as substrate.

Renal disease

Mice were monitored at 3 and 6 months of age for evidence of nephritis. Urine was collected using metabolic cages and the total amount of urinary protein was measured by a Comassie-based assay (Pierce, Rockford, IL, USA). Blood urea nitrogen was measured using a commercially available kit (Sigma Chemicals). Upon sacrifice, kidneys were fixed, sectioned and stained with hematoxylin and eosin and periodic acid schiff. At least 100 glomeruli were examined per section, by light microscopy, for evidence of inflammation, and/or tissue damage, and graded as described before (27) in a blinded fashion. The occurrence of any mesangipathic, capillary hyaline, proliferative, membranous or crescentic glomerular changes was also noted.

Western blot analysis

Splenocyte preparations from 2- to 3-month old mice were depleted of red blood cells using tris–ammonium chloride, and single cell suspensions were lysed using 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 μg ml⁻¹ leupentin, 1% Triton X-100, 1 mM phenylmethylsulphonylfluoride and 1 mM Na3VO4. Total protein was quantified by the Bradford assay, and 40 μg per lane was loaded onto SDS-PAGE gels. The following primary antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA): anti-mTOR (catalog #2972), anti-phospho-mTOR (Ser2448, catalog #2971), anti-4EB-BP1 (catalog #9452), anti-phospho-4E-BP1 (catalog #8548, catalog #8542).
Thr37/46, catalog #9459), anti-p70S6 kinase (catalog #9202), anti-phospho-p70S6 kinase (Thr389, catalog #9205), anti-AKT (catalog #9279), anti-phospho-AKT (T308, catalog # 9275), anti-phospho-IKKα (Ser180)/IKKβ (Ser181) (catalog #9574), anti-phospho-Erk-1,2 MAPK (catalog #9102) and anti-SAPK/JNK (catalog #9252). Anti-PI3K (p85, catalog # 06-497) was purchased from Upstate Biotechnology (Waltham, MA, USA), whereas the phospho-specific anti-AKT (pS473) antibody was from Biosource International (Camarillo, CA, catalog # 44-622).

Monoclonal mouse anti-glyceraldehyde-3 phosphate dehydrogenase (GAPDH), or anti-actin, from Advanced Immunochemical Inc. (Long Beach, CA, catalog # RGM2) was used to further confirm equal protein loading. HRP-conjugated secondary antibodies and the ECL-plus Detection kit (Amersham, Piscatawy, NJ, USA) were used to develop the blot. The respective band intensities were measured using ImageJ (http://rsb.info.nih.gov/ij), and normalized based on GAPDH levels. Where samples from different strains were compared, all normalized band intensities were expressed as ratios relative to the B6 levels. In some studies, lysates were made from splenic T- or B cells purified using anti-CD4 or anti-CD19 magnetic beads (Miltenyi Biotech); in these studies the resulting cells were >95% pure.

In vivo treatment protocol
RAD001 (2% w/w stock solution; Novartis, Basel, Switzerland) was diluted in PBS to 1 mg ml⁻¹. Three to 6-month-old mice were administered a final dose of 10 mg kg⁻¹ RAD001 or vehicle alone (i.e. ‘placebo’), by oral gavage 3x/week for 4 weeks. Serum and 24-h urine samples were obtained on day 0, day 14 and day 28. All serum samples were assayed for auto-antibodies by ELISA. The urine samples were assayed for total protein. On day 28, upon sacrifice, the cellular composition of the spleen and nodes were determined by flow cytometry, and the kidneys were examined for pathology, as described above. In addition, the expression of various signaling molecules in the spleens of the treated mice was assayed by western blot, as described above.

Statistics
Statistical comparisons were performed using the paired or unpaired Students’ t-test, as appropriate, using SigmaStat (Jandel scientific). For all experiments, the mean/SEM is also depicted.

Results
Cellular phenotypes in B6.Sle1b⁺,lpr mice
As depicted in Fig. 1 and Table 1, the epistatic interaction of Sle1b⁺ and FASlpr led to severe splenomegaly and

![Fig. 1](https://academic.oup.com/intimm/article-abstract/19/4/509/691749)
lymphadenopathy, with significantly elevated total lymphocyte numbers in all secondary lymphoid organs examined. The spleens exhibited significantly expanded numbers of CD3+ve, CD4–ve, CD8–ve DN T cells, relative to B6 mice bearing Sle1bz alone, or FAS<sup>a</sup> alone (Fig. 1, Table 1). In addition, both the CD4 and CD8 T cells from B6.Sle1bz<sup>lpr</sup> mice were significantly more activated, compared with the sizes and cell counts noted in the LN from the control strains (10, and data not shown). As was the case with the spleen, there was a massive expansion of DN T cells (25.9 ± 4.4% of all T cells; P < 0.005, compared with B6.Sle1bz<sup>lpr</sup> mice; data not plotted). Likewise, 22% of B6.Sle1bz<sup>lpr</sup> splenic B cells were CD23–ve, CD21–ve, and the corresponding values for B6.Sle1bz and B6.lpr splenic B cells were 12% and 19%, respectively (n = 3 each, aged 3 months; data not plotted). In the limited numbers of mice examined, no significant gender differences were noted in these cellular phenotypes.

Similar changes were also noted in the LN of these mice. Hence, B6.Sle1bz<sup>lpr</sup>-derived submandibular/brachial LN weighed ~424.2 ± 96.2 mg, with (76.0 ± 9.2) × 10<sup>6</sup> cells per node (representing the mean ± SEM of six mice, aged 6 months). These figures were 50- to 100-fold higher than the sizes and cell counts noted in the LN from the control strains (10, and data not shown). As was the case with the spleen, there was a massive expansion of DN T cells (25.9 ± 4.4% of all T cells; n = 6) and B1a cells (63.8 ± 2.0% of all B cells, n = 6) in B6.Sle1bz<sup>lpr</sup> LN, compared with the control LN.

**Functional differences in B6.Sle1bz<sup>lpr</sup> lymphocytes**

Since B6.Sle1bz<sup>lpr</sup> spleens and nodes harbored increased numbers of lymphocytes, we proceeded to ask if these differences were the consequence of reduced apoptosis, or increased cell division. For this purpose, we focused on

<table>
<thead>
<tr>
<th>Splenic T cells</th>
<th>B6 (n = 6)</th>
<th>B6.Sle1bz&lt;sup&gt;lpr&lt;/sup&gt; (n = 6)</th>
<th>B6.lpr (n = 6)</th>
<th>B6.Sle1bz&lt;sup&gt;lpr&lt;/sup&gt; (n = 6)</th>
<th>B6.Sle1bz&lt;sup&gt;lpr&lt;/sup&gt; compared with B6&lt;sup&gt;b&lt;/sup&gt;</th>
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<th>B6.lpr&lt;sup&gt;d&lt;/sup&gt;</th>
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<tr>
<td>Weight (mg)</td>
<td>133.3 ± 8.9</td>
<td>131.8 ± 10.1</td>
<td>175.0 ± 22.4</td>
<td>405.3 ± 47.4</td>
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<td>Cell count (× 10&lt;sup&gt;6&lt;/sup&gt;)</td>
<td>95.7 ± 7.3</td>
<td>103.8 ± 7.3</td>
<td>92.9 ± 5.1</td>
<td>239.2 ± 15.7</td>
<td>***</td>
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<tr>
<td>%CD4 T cells&lt;sup&gt;c&lt;/sup&gt;</td>
<td>15.0 ± 0.7</td>
<td>14.8 ± 0.6</td>
<td>13.7 ± 1.1</td>
<td>14.9 ± 1.0</td>
<td>NS</td>
<td>NS</td>
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</tr>
<tr>
<td>%CD8 T cells&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9.2 ± 1.4</td>
<td>11.2 ± 0.9</td>
<td>7.0 ± 1.1</td>
<td>7.9 ± 1.8</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
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<td>%CD3+ DN T cells&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.6 ± 0.4</td>
<td>3.8 ± 0.5</td>
<td>7.5 ± 1.5</td>
<td>17.2 ± 1.4</td>
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<tr>
<td>CD4 T cells: %CD69+ve</td>
<td>28.2 ± 2.3</td>
<td>25.7 ± 6.7</td>
<td>38.5 ± 3.2</td>
<td>50.1 ± 5.2</td>
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<td>CD4 T cells: mean FSC&lt;sup&gt;d&lt;/sup&gt;</td>
<td>73.8 ± 0.8</td>
<td>73.6 ± 1.1</td>
<td>74.1 ± 1.2</td>
<td>78.6 ± 1.4</td>
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<tr>
<td>CD8 T cells: %CD69+ve</td>
<td>17.2 ± 1.6</td>
<td>17.6 ± 4.4</td>
<td>30.6 ± 2.6</td>
<td>40.9 ± 3.9</td>
<td>***</td>
<td>*</td>
<td>NS</td>
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<tr>
<td>CD8 T cells: mean FSC&lt;sup&gt;d&lt;/sup&gt;</td>
<td>84.2 ± 1.3</td>
<td>81.7 ± 1.5</td>
<td>80.8 ± 1.9</td>
<td>84.6 ± 0.9</td>
<td>NS</td>
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<sup>a</sup>Indicated are the mean ± SEM values of the indicated parameters drawn by observing six mice per strain at the age of 6 months. This included three male and three female mice per strain.

<sup>b</sup>Indicated are the results of comparing the values obtained from the B6.Sle1bz<sup>lpr</sup> mice against the data from the three control strains using the student’s t-test (*P < 0.05; **P < 0.01; ***P < 0.001; NS = not significant).

<sup>c</sup>Indicated are the respective percentages of various T-cell subsets out of all live cells within the lymphocyte gate. DN T cells are T cells that are CD3+ve, but negative for CD4 and CD8.

<sup>d</sup>Cell size was inferred from the mean forward scatter (FSC) of the cells, as ascertained by flow cytometry.

<sup>e</sup>Indicated are the respective percentages of various B-cell subsets out of all live cells within the lymphocyte gate. B2 cells are B220+ve, whereas B1a cells are B220+ve and CD5+ve. B220+ve cells were also subanalysed based on their expression of CD23 and CD21.

**Table 1.** Activation status and lymphocyte subsets in B6.Sle1bz<sup>lpr</sup> mice

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<td>9.2 ± 1.4</td>
<td>11.2 ± 0.9</td>
<td>7.0 ± 1.1</td>
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splenic magnetic bead-purified CD4+ T cells from the four strains, at the age of 2 months. As noted in Fig. 3, B6.lpr and B6.Sle1b+/lpr T cells and T-cell blasts exhibited reduced apoptosis, following anti-CD3 stimulation, unlike Sle1b−/− and B6 T cells. In contrast, B6.Sle1b+/lpr T cells demonstrated increased cell division following anti-CD3 stimulation, compared with B6 and B6.lpr T cells (Fig. 4). Surprisingly, the T cells from B6.Sle1b+/lpr spleens were rather lethargic in response to anti-CD3 stimulation, compared with the control strains, possibly indicating that a substantial fraction of T cells from these lupus mice might have already acquired a “terminal exhausted” phenotype (following chronic stimulation by endogenous autoantigens).

**Auto-antibodies and pathology in B6.Sle1b+/lpr mice**

B6.Sle1b+/lpr mice also exhibited high serum levels of IgM and IgG auto-antibodies to ssDNA, dsDNA, histones and histone/DNA complexes (Fig. 5), compared with the B6.lpr and B6.Sle1b−/− controls. Although B6.Sle1b+/lpr mice were relatively free of auto-antibodies at this age, B6.lpr mice exhibited a modest degree of serological autoreactivity (Fig. 5), as has been noted previously (10). Also as noted previously, B6.Sle1b+/lpr females exhibited significantly higher levels of IgG anti-dsDNA ($P < 0.0005$), anti-ssDNA ($P < 0.0005$), anti-histone ($P < 0.005$), anti-glomerular antibodies ($P < 0.01$), and anti-ssDNA ($P < 0.01$), anti-histone (DNA) ($P < 0.003$) and anti-glomerular antibodies ($P < 0.05$), compared with B6.Sle1b+/lpr males (data not separately plotted). As one might have predicted, B6.Sle1b+/lpr mice also developed nephritis as gauged by various parameters. Although the increased proteinuria and azotemia in B6.Sle1b+/lpr mice failed to attain statistical significance, the histological glomerulonephritis (GN) scores in these mice were significantly higher than those in all control groups (Fig. 6A–C). In resonance with the gender difference in auto-antibody profiles, B6.Sle1b+/lpr females exhibited significantly more severe renal disease (average GN score = 2.8, $P < 0.05$), compared with B6.Sle1b+/lpr males (average GN score = 1.9; data not plotted separately). Finally, B6.Sle1b+/lpr mice exhibited accelerated mortality compared with the controls, again with a distinct gender difference (Fig. 6D; $P < 0.001$).

**Hyperactivated PI3K/AKT/mTOR axis in B6.Sle1b+/lpr mice**

Given that several genetically engineered mouse models with exaggerated PI3K/AKT signaling activity have been noted to develop ALPS with features similar to those observed in B6.Sle1b+/lpr mice (11, 12, 17, 18), we next examined the latter strain for evidence of increased signaling via this axis. For these studies, females were used for all strains since they exhibited more severe disease. As depicted in Fig. 7A, significantly increased pAKT levels were noted in B6.Sle1b+/lpr spleens compared with the spleens of all three control strains, even after controlling for the levels of total AKT. Given that the epistatic interaction of Sle1b−/− and FAS+/− led to significantly elevated pAKT levels, we then analyzed these mice for the levels of an important downstream target of pAKT—mTOR. As is evident from Fig. 7(B), B6.Sle1b+/lpr spleens exhibited significantly elevated phosphorylated mTOR levels relative to the levels of total mTOR. Additionally, another downstream target, caspase 9, was also hyperactivated in B6.Sle1b+/lpr spleens (data not shown). The expression levels of downstream molecules activated by mTOR were next examined. As depicted in Fig. 7(C), both the expression of phospho-p70S6K and phospho-4EBP-1 were elevated in B6.Sle1b+/lpr spleens. Whereas the B6.Sle1b−/− and B6.lpr mice also showed increased phosphorylation of p70S6K, relative to B6, the elevated levels of total/phosphorylated 4EBP-1 were noted to be particularly dependent upon the epistatic interplay of Sle1b+/− and FAS+/− (Fig. 7C).

The above studies indicate that several downstream signaling rami controlled by pAKT were hyperactivated in B6.Sle1b+/lpr mice. We next examined the expression levels of ‘upstream’ molecules (PI3K and PTEN) that may potentially modulate pAKT activity. Activated PTEN levels were significantly reduced in B6.Sle1b−/− and B6.lpr spleens (relative to B6 controls), with the levels tending to be even lower in B6.Sle1b+/lpr mice (Fig. 7D). Likewise, elevated PI3K levels were noted in B6.Sle1b+/lpr spleens, but these levels did not appear to be significantly higher than the levels in either control strains, Sle1b−/− or FAS+/−. Presently, it is not clear if the monitoring of particular activated forms of PI3K or PTEN might uncover any epistatic impacts of Sle1b+/− and FAS+/− on PI3K/PTEN balance. Although the above western blot studies
were conducted with whole spleens, the AKT axis also appeared to be activated in magnetic bead-purified CD4+ T- and B cells from B6.Sle1bz.lpr spleens, compared with control CD4+ T- and B cells (Fig. 7E). Notably, this axis was observed to be particularly hyperactivated among the CD4 T cells from B6.Sle1bz.lpr spleens (Fig. 7E).

Blocking mTOR modulated disease in B6.Sle1bz.lpr mice

Given that the PI3K/AKT/mTOR signaling axis was overactive in B6.Sle1bz.lpr mice, we asked if pharmacological modulation of this pathway might ameliorate lymphoproliferative lupus. mTOR is a key downstream effector in the PI3K/AKT signaling axis (21, 28) and the phosphorylated form of this molecule is evidently hyperexpressed in B6.Sle1bz.lpr mice. Importantly, targeting this molecule using rapamycin has been shown to ameliorate various forms of malignancies (28–31). Recently, a more effective derivative of rapamycin, RAD001, with better pharmacokinetic properties has been described (32–35). Three- to 6-month old B6.Sle1bz.lpr mice that had already developed features of lymphoproliferative lupus were treated with RAD001, or placebo, to gauge the impact of targeting this specific signaling axis, once lymphoproliferative autoimmunity had already set in.

The most dramatic effect of RAD001 treatment was the rapid mitigation of the lymphoproliferative phenotypes in B6.Sle1bz.lpr mice, noted in at least two independent treatment studies that were conducted. This was marked by the rapid and significant reduction of the spleen and nodes, both in terms of weight and total cell numbers (Fig. 8A). One of the cardinal features that mark the secondary lymphoid organs of B6.Sle1bz.lpr mice is the massive expansion of two peculiar cell populations: CD3+, CD4−/CD8− DN T cells, as well as CD5+ve B1a cells, as described above (Table 1, Fig. 1). Importantly, treatment of B6.Sle1bz.lpr mice with RAD001 significantly reduced splenic DN T cells and normalized the skewed B1a:B2 ratios in a reproducible fashion (Fig. 8A). Similar findings were noted in the LNs as well (data not plotted). Full blood counts on these mice revealed only a reduction in lymphocytes but not other cell types or the hemoglobin levels (Fig. 8B). Surprisingly, however, substantial increases were noted in the absolute numbers of platelets, basophils and neutrophils, the significance of which presently remains unknown (Fig. 8B).

The impact of RAD001 treatment on the serological phenotypes was more subtle. When one compared the average levels of serum IgG anti-nuclear antibodies (ANA),

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**Fig. 3.** Impact of Sle1bz and FAS<sup>z</sup> on lymphocyte apoptosis. (A) Purified primary splenic CD4 T cells from the four strains (n = 6 mice each) were stimulated with plate-bound anti-CD3 antibody for 24 h and 48 h and assessed for apoptosis using Annexin V and 7-AAD. Plotted are percentage of CD4 T cells positive for both Annexin V and 7-AAD. (B) Splenic T cells were stimulated with soluble anti-CD3 for 48 h, then Ficoll-purified and re-stimulated with plate-bound anti-CD3. Apoptosis was assessed 24 h or 48 h later. Depicted are two independent experiments (n = 3 each) showing the percentage of apoptotic cells positive for both Annexin V and 7-AAD among the T-cell blasts 24 h after re-stimulation. Shown P values pertain to Student’s t-test comparison of apoptotic rates of B6.Sle1bz.lpr T cells against that of T cells from the other strains.
Impact of Sle1b<sup>z</sup> and FAS<sup>ab</sup> on lymphocyte division. Purified primary splenic CD4 T cells from the four strains (n = 3 mice each) were CFSE labeled, stimulated with plate-bound anti-CD3 antibody for 96 h and assessed for the degree of cell division based on CFSE dilution. The 1-D histograms plotted are representative of three mice for each strain. The percentage of divided cells at 96 h were 46%, 35% and 35% for B6, 58%, 56% and 38% for B6.lpr, 64%, 68% and 70% for B6.Sle1b<sup>z</sup> and 34%, 36% and 18% for B6.Sle1b<sup>z</sup>.lpr T cells.

**Fig. 4.**

Status of other signaling axes in B6.Sle1b<sup>z</sup>.lpr mice

Finally, it became apparent that the PTEN/P3K/AKT axis was not the only pathway that was hyperactivated in B6.Sle1b<sup>z</sup>.lpr spleens. Among several other signaling pathways examined, the most dramatic difference was noted in the expression levels of Erk1,2 MAPK and the NFKB pathway (based on the increased levels of pIKK<sub>ab</sub>), as depicted in Fig. 9(B). It is now clear that there is a substantial degree of crosstalk between these other signaling axes and the AKT pathway. Importantly, one of the downstream targets of phosphorylated mTOR is IKK<sub>ab</sub>, and this might explain its increased phosphorylation in B6.Sle1b<sup>z</sup>.lpr mice. As a test of how independent other signaling pathways may be in the spleens of these mice relative to the AKT axis, additional signaling pathways were re-examined after RAD001 treatment. As demonstrated in Fig. 9(C), RAD001 treatment suppressed both pIKK<sub>ab</sub> and Erk1,2 significantly with the impact on the former being more profound.

**Discussion**

Given that B6.Sle1<sup>v</sup>.lpr mice, but not B6.lpr controls, exhibited ALPS, we had earlier reasoned that in addition to the genetic aberration in FAS, an additional genetic event as exemplified by the NZM2410/NZW 'z' allele of Sle1 may be required for full-blown lymphoproliferative lupus to ensue (10). However, the Sle1 genetic interval itself is comprised of several genetic players including Sle1a, Sle1b and Sle1c (24). The present work illustrates that the Sle1b locus within this susceptibility interval bearing the NZM2410/NZW 'z' allele of the SLAM family of molecules may be sufficient and necessary for full-blown lymphoproliferative lupus, in the context of aberrant FAS function. This is consistent with the notion that Sle1b may harbor the most 'potent' gene within the Sle1 interval, and confirms earlier reports indicating the pathogenic relevance of this locus (24, 36). The strain reported in this communication harbors the Sle1b<sup>z</sup> locus, but excludes the Sle1a and Sle1c loci.

The present report illustrates that the clinical features of lymphoproliferative lupus in B6.Sle1b<sup>z</sup>.lpr mouse appear to arise as a consequence of the epistatic interplay of Sle1b<sup>z</sup> with FAS<sup>ab</sup>. This includes the splenomegaly, lymphadenopathy, increased numbers of DN and CD4+ T cells, B1a cells, anti-nuclear and anti-glomerular auto-antibodies, as well as renal disease. However, the mechanistic contributions of the two genetic players appear to be very different—whereas FAS<sup>ab</sup> (but not Sle1b<sup>z</sup>) impairs apoptosis of activated mature lymphocytes (7, 23; Fig. 3), Sle1b<sup>z</sup> appears to impede the censoring of immature lymphocytes (37), and facilitate the proliferation of mature lymphocytes (Fig. 4). Hence, the expanded numbers of lymphocytes and the other epistatic phenotypes observed in B6.Sle1b<sup>z</sup>.lpr mouse appear to be the consequence of Sle1b<sup>z</sup> and FAS<sup>ab</sup> acting upon reciprocal checkpoints controlling lymphocyte homeostasis.

Literature reports have amply demonstrated that overactivation of the PI3K/AKT signaling cascade invariably leads to lymphoproliferative lupus. Perhaps the closest resemblance to the B6.Sle1<sup>v</sup>.lpr and B6.Sle1b<sup>z</sup>.lpr strains is the PTEN<sup>−/−</sup> haploinsufficient strain (11). PTEN encodes a phosphatase that is mutated or aberrantly expressed in a high percentage of human tumors (15, 16). Although homozygous deficiency of PTEN leads to embryonic lethality, B6/129.PTEN<sup>−/−</sup>...
haploinsufficient mice develop severe lymphadenopathy prominently affecting the submandibular, axillary and inguinal LN, and autoimmunity, similar to the features observed in B6. Sle1bzlpr mice. PTEN haploinsufficient mice also develop high titers of IgG ANAs and severe GN with a rapid time course. Finally, PTEN+/−C0 mice also exhibit an expanded B1a cell population (11, 19), akin to that observed in B6. Sle1bzlpr mice. Likewise, mice that hyperexpress PI3-kinase or phosphorylated AKT also exhibit similar phenotypes (17, 18). Given that PTEN and PI3K reciprocally regulate pAKT activity, the above reports collectively indicate that excessive activity via the PI3K/AKT signaling axis invariably precipitates ALPS. In addition, it is clear that the upregulation of this signaling axis in B and T cells can lead to the expansion of B1 cells and DN T-cells (11, 12, 17, 19), respectively, representing the cell populations that were prominently expanded in B6. Sle1bzlpr mice. The present study demonstrates that this signaling axis is also hyperactivated in B6. Sle1bzlpr mice, as a consequence of the epistatic end-product of two genetically distinct elements. This is supported by the elevated pAKT levels and the activated forms of its downstream targets, mTOR and caspase 9 (data not shown) in B6. Sle1bzlpr, relative to the B6, B6. Sle1bZ and B6. lpr control strains. The notion that the hyperexpressed mTOR molecule is also functionally active is supported by the elevated phosphorylation of its downstream targets, 4EBP-1 and p70S6K in B6. Sle1bzlpr spleens. Our preliminary studies suggest that different

\[\text{Fig. 5.} \] High titers of anti-nuclear and anti-glomerular antibodies in B6. Sle1bzlpr mice. Indicated are the serum auto-antibody levels in 6-month-old B6 (n = 6), B6. Sle1bZ (n = 6), B6. lpr (n = 6), B6. Sle1bZ/lpr mice (labeled as B6. Sle1bZ/lpr; n = 11–19). Each dot represents data obtained from an individual mouse and the horizontal bar indicates the mean value for each group. In all groups, equal numbers of males and females were used; the latter exhibited significantly higher levels of auto-antibodies, as detailed in the text. ²Student’s t-test P value compared with the B6 (−− control) levels. ¹Student’s t-test P value compared with the B6. Sle1bZ/lpr (++ control) levels.

\[\text{Fig. 6.} \] Increased renal disease and mortality in B6. Sle1bZ/lpr mice. Depicted are the 24-h urinary protein excretion rates (A), blood urea nitrogen (B) and GN scores (C) noted in 6-month-old B6 (n = 6–8), B6. Sle1bZ (n = 6–8), B6. lpr (n = 6) and B6. Sle1bZ/lpr mice (labeled as B6. Sle1bZ/lpr; n = 11–19). Each dot represents data obtained from an individual mouse and the horizontal bar indicates the mean value for each group. Wherever the B6. Sle1bZ/lpr values differed significantly from the control values, the corresponding P values are indicated below the respective control strains. Whereas the control strain (n = 45, in total, comprised of 20 B6, 10 B6. lpr and 15 B6. Sle1bZ mice) exhibited no mortality till 12 months of age, B6. Sle1bZ/lpr mice (n = 42 males and 43 females) exhibited significantly greater mortality (Chi-square test P value < 0.001; D). B6. Sle1bZ/lpr females exhibited significantly accelerated mortality compared with B6. Sle1bZ/lpr males (P < 0.001).

AKT/mTOR axis in lymphoproliferative lupus
Fig. 7. Expression of various signaling molecules in splenic lysates. Total lysates from 2-month-old B6, B6.Sle1b−/−, B6.lpr and B6.Sle1b−/− (labeled as B6.Sle1b.lpr) spleens (n = 3–5, per strain), were electrophoresed, blotted and probed with antibodies to pAKT or total AKT (A), p-mTOR or total mTOR (B), p4EBP1 or total 4EBP-1 (C), phospho-p70S6K or total p70S6K (C), as well as PI3K (p85), PTEN and pPTEN (D). Depicted on the left are representative blots from the four strains studied. Plotted on the right are mean ± SEM expression levels of three to five independent spleens per strain, expressed as ratios. For the total levels of signaling molecules, ratios were derived relative to the GAPDH levels; for the phosphorylated forms of the proteins, ratios were derived by dividing the levels of the phosphorylated form by the total levels of that molecule in the same lysate. Indicated below each strain are the statistical P values comparing the expression levels in that strain, to the corresponding levels in B6 spleens, as determined using the Student’s t-test (*P < 0.05; **P < 0.01; ***P < 0.001). Depicted in (E) are the levels of pAKT in magnetic bead-purified B cells or CD4+ T cells from 2-month-old B6 or B6.Sle1b−/− spleens (n = 3 independent spleens per group), expressed as ratios, relative to the actin content (left) or total AKT (right). Each dot represents data obtained from an individual mouse and the horizontal bar indicates the mean value for each group. Shown experiments are representative of two to three studies each.
Ly108 isoforms, which constitutes a key candidate gene within \( \text{Sle1b} \) (37), may impact AKT activation differentially (unpublished observations); in contrast, the contribution of \( \text{FASlpr} \) to this axis may be indirect, arising as a consequence of increased numbers of activated (potentially autoreactive) lymphocytes in the periphery that have failed to be effectively removed. Finally, the additional gender differences observed in the phenotypes of B6.\( \text{Sle1bz.lpr} \) mice may well relate to how female sex hormones impact signaling via the AKT axis, as has been demonstrated in other model systems (38–40).

Given that the PI3K/AKT/mTOR axis is fired up in B6.\( \text{Sle1bz.lpr} \) mice, this provides an attractive target for therapeutic intervention in this disease. In theory, one could modulate this axis at several nodes—PI3K, pAKT, mTOR or any of the downstream mediators. Although wortmanin and Ly29400 have worked satisfactorily as PI3K inhibitor \textit{in vitro}, they have not proven to be suitable for clinical therapeutics for a variety of reasons, including poor solubility and relative non-selectivity. Indeed, our initial attempts to use Ly29400 as a therapeutic option were not successful due to the poor solubility of this compound (Patel and Mohan, unpublished observations). Although the blocking of pAKT appears to be a reasonable target, the use of selective AKT inhibitors has been infrequently reported. In contrast, mTOR represents a very attractive target for several reasons, as discussed elsewhere (28–35).

**Fig. 8.** Blocking mTOR function and its impact on lymphoproliferative autoimmunity. The spleen and lymph node sizes and cellular composition (in terms of absolute cell numbers) were compared between the RAD001-treated mice and placebo-treated B6.\( \text{Sle1bz.lpr} \) mice, at the conclusion of the study on D28 (A, \( n = 6–8 \) mice per group). The values in the RAD001 group were expressed as a percentage of the values in the placebo group. Their full blood count profiles were similarly compared (B). The \( P \) values shown on the right in (A) and (B) pertain to Student's \( t \)-test comparisons of the data obtained from the two groups. Plotted in (C) are the serum IgG auto-antibody levels in the experimental (bottom) and placebo (top) groups of mice on day 0 and day 28 (connected by dotted lines) of the treatment protocol, with six to seven mice included per group. Shown \( P \) values pertain to Student's paired \( t \)-test comparisons of the day 0/day 28 data within each group. Treatment of 4- to 6-month old B6.\( \text{Sle1bz.lpr} \) mice with RAD001 was not associated with as significant a rise in IgG auto-antibodies within individual mice, when compared with the serological changes observed in the placebo-treated mice (C). Shown data in (A)–(C) is representative of two independent experiments with similar results. Depicted in (D) are the 24-h urinary protein excretion rates in the RAD001-treated mice and the placebo-treated mice on day 0 and day 28, pooled from two independent experiments.
p70S6K is critical for translation of several ribosomal proteins, as well as components of the translational machinery. Although the blocking of mTOR function was noted to have only a marginal impact on total protein synthesis in normal cells, it has been observed to have a profound impact on malignant cells (29, 31). Moreover, blocking mTOR function has been observed to ameliorate the lymphoproliferative phenotypes attributed to PTEN haploinsufficiency, as well as AKT overactivity, confirming that the disease triggered by aberrant PTEN/AKT function may be critically dependent upon mTOR activation (21, 29, 41, 42).

Several pharmaceutical companies have manufactured mTOR inhibitors, a couple of which are presently in clinical trials for cancer treatment. Rapamycin was originally isolated from cultures of *Streptomyces hygroscopicus* sampled on Easter Island ('Rapa Nui') and has also been accorded the brand name, Rapamune (sirolimus). Rapamycin derivative, including RAD001 (everolimus, Novartis), CCI-779 (Wyerth) and AP23573 (Ariad Pharmaceuticals), have been demonstrated to be particularly effective in treating various malignancies (29, 34, 35, 42). Indeed, it was remarkable to note that the administration of RAD001 to B6.Sle1b.lpr mice was able to mitigate most of the examined phenotypes, despite beginning the treatment after disease onset, with the effect on lymphoproliferation being more dramatic compared with the impact on autoimmunity. It is conceivable that if therapy

![Fig. 9](https://academic.oup.com/intimm/article-abstract/19/4/509/691749/1081749)
had been commenced prior to the onset of disease, or if more prolonged therapy had been instituted, both features of lymphoproliferative autoimmunity might have been ameliorated more profoundly. In this regard, we have recently observed that commencing RAD001 therapy early in disease and continuing therapy for 2 months (in another strain of murine lupus) profoundly dampened autoimmunity as well as lymphoproliferation (Wu et al., in preparation).

The observation that RAD001 treatment had significant, but somewhat limited impact on parallel signaling pathways also activated in ALPS (Fig. 9), suggests that the pharmacological blocking of additional signaling axes may yield augmented therapeutic benefit. In this context, it has been reported that intermittent dosing and combination therapy with other modulators may boost the eventual clinical usefulness of mTOR-targeted therapy in cancer (35, 20). Finally, this treatment regime was not accompanied by any gross side effects, including weight loss, premature death or alterations in blood counts (data not shown).

The B6.Sle1b♂.fpr model of ALPS has important parallels to human ALPS. Besides the strong phenotypic resemblance, both diseases may also share common genetic origins. Aberrations in FAS have also been implicated in human ALPS (1–4, 8, 9). Moreover, the genetic interval on human chromosome 1 that is syntenic to murine Sle1b has also been implicated in human lupus (43–45). Given that both the genetic components studied in this communication may be at play in human disease as well, the lessons learned using this mouse model are likely to be of relevance in understanding human lymphoproliferative disease as well. One may predict that this axis may be hyperactivated in human ALPS as well as in subsets of lupus patients harboring lymphoid malignancies (46–48). It remains to be determined if this signaling axis might also be up-regulated in lupus patients without any evidence of lymphoproliferation. Clearly, a detailed workout of the signaling pathways in phenotypically well-defined subsets of lupus/ALPS patients is warranted before this axis can be therapeutically targeted in patients.

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Disclosures
The authors have no financial conflict of interest.

Abbreviations

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<th>Abbreviation</th>
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<tr>
<td>ALPS</td>
<td>autoimmune lymphoproliferative syndrome</td>
</tr>
<tr>
<td>DN</td>
<td>double negative</td>
</tr>
<tr>
<td>GN</td>
<td>glomerulonephritis</td>
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<td>LN</td>
<td>lymph node</td>
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References


