Genetic evidence for the role of Erk activation in a lymphoproliferative disease of mice

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Germline mutation of the linker for activation of T cells (LAT) gene at the phospholipase C-γ1 (PLC-γ1)-binding site leads to a fatal lymphoproliferative disease in mice. The hyperactivated T cells that develop in these mice have defective T-cell antigen receptor (TCR)-induced calcium flux but enhanced mitogen-activated protein kinase (MAPK) activation. We used genetic analysis to investigate genes whose products might suppress MAPK activation and lymphoproliferative disease in LAT mutant mice. B-lymphocyte adaptor molecule of 32 kDa (Bam32) is a known mediator of MAPK activation in B cells. We recently reported that in CD4$^+$ T cells, Bam32 deficiency decreased MAPK activation and specifically extracellular-signal-regulated kinase (Erk) signaling, following TCR stimulation. By crossing the Bam32 null mutation onto the LAT knock-in background, we found that the Bam32 null mutation delayed the onset and decreased the severity of lymphoproliferative disease in LAT knock-in mice. The pulmonary lymphocyte infiltration seen in LAT knock-in mice was also markedly decreased in double-mutant mice. Additionally, Erk activation was diminished in LAT knock-in Bam32 knockout CD4$^+$ T cells. To more accurately determine the role of Erk in this delay of lymphoproliferative disease, we also bred a transgenic, hypersensitive Erk allele (the Erk2 sevenmaker mutant) onto the LAT knock-in Bam32 knockout double-mutant background. These triple transgenic mice demonstrated a role for Erk activation in lymphoproliferative disease caused by the LAT knock-in mutation.

Bam32 | LAT | lymphoproliferation | T cells

Linker for activation of T cells (LAT) is an adaptor protein that is phosphorylated by the protein tyrosine kinase ZAP-70 following T-cell antigen receptor (TCR) engagement (1, 2). Following phosphorylation of the distal four tyrosines of LAT, several other adaptors and enzymes can bind to LAT directly, such as Grb2, Gads, and PLC-γ1, or indirectly, such as SLP-76, Cbl, Nck, Wasp, and Vav (3). LAT plays an important role both in TCR signaling in mature T cells and during T-cell development. LAT-deficient mice show a complete block in thymocyte development at the early CD4$^-$CD8$^-$ stage, and no mature αβ T cells are present in these mice (4). Mutation of the distal four tyrosines of LAT to phenylalanines (4YF) blocks TCR-mediated calcium mobilization, extracellular signal-regulated kinase (Erk) activation and nuclear factor of activated T-cells (NFAT) activation in Jurkat T cells (1, 5). Mice that have the 4YF LAT mutation incorporated into the germline show an identical phenotype to LAT-deficient mice (6). In vitro studies show that the single point mutation of human LAT Y132F blocks binding of PLC-γ1 to LAT (7). In mice, this point mutation at Y136 (the mouse equivalent of Y132) results in a partial block in early T-cell development. However, over time, a fatal lymphoproliferative disease characterized by expansion of Th2 helper CD4$^+$ T cells is observed in LAT-Y136F mice (8, 9). In addition, LAT-Y136F mice have multi-organ infiltration of lymphocytes and hallmarks of autoimmune disease including autoantibody production and glomerular IgG deposits in kidney (8–11). In LAT-Y136F mice, natural regulatory T-cell development is strongly suppressed (11, 12), and CD4$^+$ T cells show resistance against TCR-induced cell death (8). However, the exact mechanisms mediating CD4$^+$ T-cell hyperproliferation in LAT-Y136F mice are not well understood.

Erk is a serine-threonine mitogen-activated protein kinase (MAPK) that is known to be critical for proliferation in many systems (13). Erk activation is also known to be important for T-cell growth and differentiation (14). In T cells, Erk can be activated downstream from LAT in several ways (7). One major pathway leading to Erk activation is through PLC-γ1. Phosphorylation of LAT leads to recruitment and activation of PLC-γ1 at LAT Y136. PLC-γ1 activation results in inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) production. IP3 production stimulates release of calcium from intracellular stores. DAG activates several signaling proteins including Ras guanyl nucleotide-releasing protein (RasGRP) (15, 16). RasGRP acts as a guanine nucleotide exchange factor (GEF) to activate Ras, which then initiates a plethora of cellular activation events including stimulation of Erk through Raf and MEK kinases (17, 18). Activated Erk moves to the nucleus, where it mediates phosphorylation of a variety of substrates including transcription factors that participate in T-cell activation. An additional pathway for Erk activation in T cells is through recruitment of Grb2 to LAT, which in turn recruits and activates the Ras GEF son of sevenless (Sos) leading to Ras, Raf, Mek, and Erk activation (19, 20). Recent evidence suggests that both Sos and RasGRP pathways are needed for efficient Ras activation leading to a well-regulated and appropriate T-cell response to antigen (21, 22). The abundant T cells present in LAT-Y136F mutant mice do not show PLC-γ1 activation, but do show Erk activation. It would be unexpected for Erk activation to be mediated by RasGRP in LAT-Y136F T cells, since PLC-γ1 activation and calcium flux are abrogated in these cells, and RasGRP is known to be activated by calcium and DAG. The possibility that Grb-2/Sos mediates activation of Erk in LAT-Y136F T cells is currently under investigation. In this study, we sought to explore other pathways that might lead to Erk activation in LAT-Y136F T cells.

In B cells, B-lymphocyte adaptor molecule of 32 kDa (Bam32) plays a role in B-cell antigen receptor (BCR)-induced activation of Erk and in B-cell proliferation (23). Bam32 is an adaptor protein discovered in B cells that is also expressed in T cells (23, 24). Although the role of Bam32 in B cells is increasingly well defined, its role in T cells is largely unknown. We recently reported that Bam32 knockout mice have normal T-cell development, but defective proliferation and interleukin-4 (IL-4)

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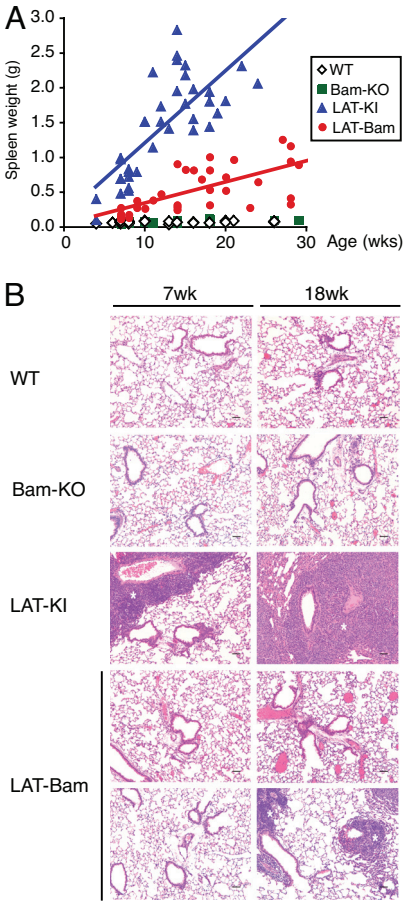

Fig. 1. Loss of Bam32 markedly improves lymphoproliferative disease in LAT-KI mice. (*A*) Spleen weights of individual mice killed at various ages in weeks (wks) are depicted. Trend lines of mutant mice are as follows: LAT-KI, $y = 0.1038x + 0.1542$ (blue line) and LAT-Bam, $y = 0.0303x + 0.0319$ (red line). (*B*) Photomicrographs of representative hematoxylin and eosin-stained tissue sections of lungs from 7- and 18-week-old WT, Bam-KO, LAT-KI, and LAT-Bam mice are shown. LAT-KI mice exhibit pronounced lymphoproliferative disease within the lungs (*) even at 7 weeks, whereas LAT-Bam mice have histologically unremarkable lungs at this age. By 18 weeks of age, LAT-KI mice exhibit large, coalescing regions of lymphoproliferative disease, which obscures the normal pulmonary parenchyma (*). In contrast, only two of 14 LAT-Bam mice exhibited any lymphoproliferative disease in the lungs. (Scale bars, 50 μm.)

production in mature CD4$^+$ T cells. Moreover, Bam32-deficient CD4$^+$ T cells had decreased Erk phosphorylation after suboptimal TCR stimulation (25). By interbreeding LAT knock-in and Bam32 knockout mice, we show that Bam32 deficiency delays progression of lymphoproliferative disease and multi-organ infiltration of lymphocytes in LAT-Y136F mice as well as decreases the level of Erk activation in LAT-Y136F T cells. We also show that modulation of Erk activation specifically is crucial for regulation of lymphoproliferative disease in LAT-Y136F mice by introduction of a hypersensitive Erk2 transgene into LAT-Y136F Bam 32 knockout mice.

Results

A hallmark of lymphoproliferative disease in LAT-Y136F (LAT-KI) mice is massive enlargement of peripheral lymphoid organs (8, 9). As a rough measure of lymphoproliferative disease, we measured spleen weight over time in LAT-KI mice. A marked increase in splenomegaly was evident in LAT-KI mice (Fig. 1*A*). We investigated a possible role for Bam32 in LAT-KI lymphoproliferative disease by generating LAT-KI Bam32 knockout double-mutant (LAT-Bam) mice. Both LAT-KI and LAT-Bam mice showed an increase in splenomegaly over time, however the rate was lower in LAT-Bam than in LAT-KI mice (Fig. 1*A*). Histological examination of spleens from LAT-KI mice showed disorganized white pulp lymphoid expansion with concomitant loss of red pulp. The degree of disorganization and lymphoid expansion seen in LAT-Bam spleens was more moderate than in LAT-KI spleens (Fig. S1). Furthermore, most LAT-KI mice died before the age of 6 months, whereas LAT-Bam mice had longer survival times than LAT-KI mice. These data indicate that Bam32 deficiency significantly modulates and delays lymphoproliferative disease in LAT-KI mice.

Another striking feature of LAT-KI mice is lymphocyte infiltration into multiple organs, especially lung (8–10). Areas of perivascular infiltration and proliferation of lymphocytes were evident in lungs from 7-week-old LAT-KI mice and became more extensive over time (Fig. 1*B*). In contrast, most LAT-Bam mice showed histologically normal lungs even at 18 weeks of age and older. In LAT-Bam mice, three of three mice showed histologically normal lungs at 7–8 weeks of age and only two of 14 mice showed obvious trafficking and proliferation of lymphocytes in lungs at greater than 16 weeks of age (Fig. 1*B*). Therefore, lymphocyte infiltration into lung, as well as lymphocyte expansion in peripheral lymphoid organs of LAT-KI mice, was markedly diminished by introduction of the Bam32 null mutation into LAT-KI mice.

LAT-KI mice accumulate striking numbers of peripheral CD4$^+$ T cells (8, 9). When comparing actual numbers of CD4$^+$ T cells from lymph nodes and spleens of 7-week-old mice, LAT-KI mice had significantly elevated numbers of CD4$^+$ T cells, whereas the numbers of CD4$^+$ T cells in LAT-Bam mice were only slightly elevated above normal (Fig. 2*A*). At 14 weeks of age (and older), LAT-Bam mice accumulated more CD4$^+$ T cells in the periphery, but still had fewer CD4$^+$ T cells than age-matched LAT-KI mice. Therefore, CD4$^+$ T-cell expansion was suppressed or, at least, delayed by Bam32 deficiency.

T-cell development is profoundly affected in LAT-KI mice (8, 9). Thymocyte numbers from LAT-KI mice are about one-tenth of normal, and T-cell development is dramatically (but not completely) blocked at the DN (CD4$^-$CD8$^-$) stage. As shown in Fig. 2*B*, Bam32 deficiency did not change the thymocyte number or the CD4CD8 subset composition of wild-type (WT) or LAT-KI thymi. In the periphery, LAT-Bam mice possessed CD4$^+$ T cells that were phenotypically similar to LAT-KI CD4$^+$ T cells, although they were found in lower numbers and percentages (Fig. 2 and Fig. S2). These results indicate that Bam32 deficiency did not alter the partial block in LAT-KI thymocyte development and that Bam32 deficiency delayed but did not prevent LAT-KI CD4$^+$ T cell expansion in the periphery.

LAT-KI peripheral CD4$^+$ T cells appear hyperactivated as assessed by characterization of several cell surface markers (8, 9), and expression of these markers was also examined in LAT-Bam CD4$^+$ T cells. TCRβ and CD3ε levels were lower than normal in LAT-KI and LAT-Bam CD4$^+$ T cells (Fig. S2). CD44^{hi}CD62L^{lo} CD4$^+$ T cells are considered "memory" or previously activated helper T cells (26) and were previously described as abundant in lymphoid tissues of LAT-KI mice (8, 9). CD4$^+$CD44^{hi}CD62L^{lo} T cells were also found in abundance in LAT-Bam peripheral lymphoid tissue (Fig. S2). We also examined expression of CD69, a marker of early T-cell activation whose up-regulation is thought to be dependent on the Ras/Erk signaling pathway (27, 28). The percentage of CD4$^+$ T cells with CD69 up-regulation was higher in LAT-KI than in WT mice, and the percentage in LAT-Bam mice was intermediate between WT and LAT-KI mice (Fig. S2). Therefore, whereas most cell surface markers were similar in showing hyperactivation of CD4$^+$ T cells, CD69 was unique among the markers that we examined in being differentially expressed in LAT-KI and LAT-Bam CD4$^+$ T cells.

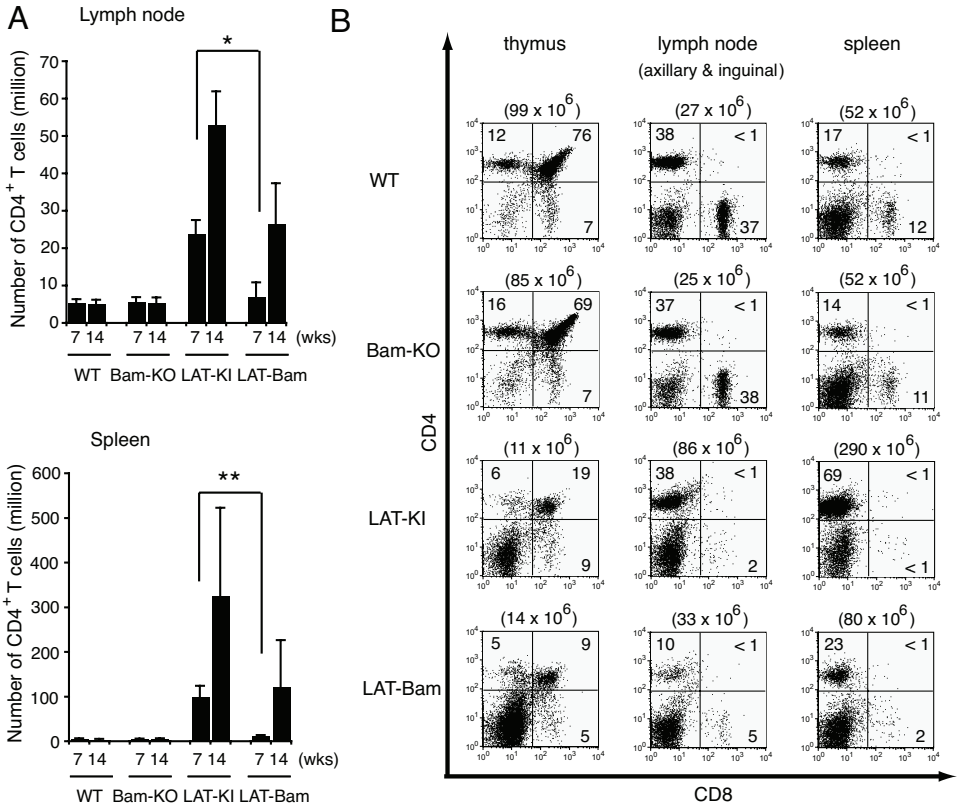

Fig. 2. CD4$^+$ T-cell proliferation is delayed in LAT-Bam compared to LAT-KI mice. (*A*) CD4$^+$ lymph node T cells and CD4$^+$ splenocytes were enumerated from lymph node (axillary and inguinal), and splenic single-cell suspensions and were analyzed by flow cytometry. Data are averages plus standard deviations (three mice per group). *P* values were calculated by student's *t*-test for unpaired data with equal variance. *, $P < 0.05$; **, $P < 0.01$. (*B*) Cell numbers and CD4/CD8 profiles in thymus, lymph nodes (axillary and inguinal), and spleen. Single-cell suspensions from 7-week-old mice were analyzed by flow cytometry. Total cell numbers are shown above each dot plot. Percentages of each quadrant are shown within each dot plot. Each dot plot shown is representative of at least three independent experiments.

T cells from LAT-KI mice show a marked shift toward Th2 differentiation as evidenced by several criteria including in vitro production of IL-4 upon PMA and ionomycin treatment, detection of serum IgG1 and IL-4 in older mice and the presence of tissue eosinophilia (8, 9). We measured serum IL-4 as an indicator of Th2 differentiation in LAT-KI and LAT-Bam mice (Fig. S3 and *SI Methods*). Serum IL-4 was detected in younger (7- to 8-week-old) LAT-KI mice but not in age-matched LAT-Bam mice. Older LAT-Bam mice (6 months old) showed similar serum IL-4 levels to younger (15-week-old) LAT-KI mice. However, LAT-Bam CD4$^+$ T cells were already differentiated into IL-4-producing cells by 7 weeks of age, as shown by intracellular flow cytometry, although serum IL-4 was not detected at this age. Therefore, T cells from LAT-Bam mice show a similar shift toward Th2 differentiation as T cells from LAT-KI mice, but IL-4 production (as measured in serum) was delayed.

We previously reported that LAT-KI T cells proliferate in vivo at a higher rate than WT T cells (8). Studies of Bam32 knockout mice showed that Bam32 is a positive regulator of in vitro proliferation of both B and T cells (23, 25). To examine the in vivo proliferative capacity of LAT-Bam T cells, mice were injected with BrdU and lymph node CD4$^+$ T cells were examined by flow cytometry. As reported previously (8), LAT-KI mice had a higher proportion of actively proliferating CD4$^+$ T cells than WT mice (Fig. 3). Proliferation rates in LAT-KI and LAT-Bam mice decreased with age. At a time when spleen weights were approximately equivalent (5- to 6-week-old LAT-KI mice, average spleen weight 0.39 g, and 12-week-old LAT-Bam mice, average spleen weight 0.26 g), LAT-KI mice showed a higher in vivo proliferation rate than LAT-Bam mice (27% vs. 16%). In vivo proliferation of T cells from younger (5- to 6-week-old) LAT-Bam mice could not be assessed because of a paucity of peripheral T cells. Therefore, when comparing proliferative rates from mice at similar points of lymphoproliferative disease

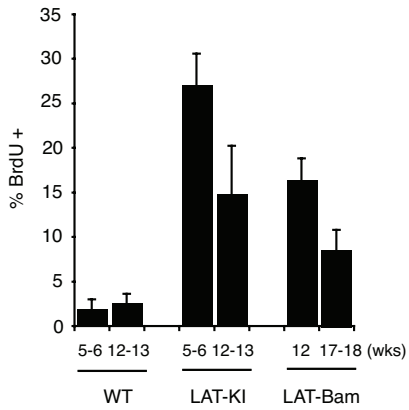

Fig. 3. The proliferation phenotype of LAT-Bam T cells. WT, LAT-KI, and LAT-Bam mice were injected i.p. with 0.8 mg BrdU at 0 and 6 h, and percentage BrdU$^+$ cells among lymph node CD4$^+$ T cells was measured by flow cytometry 24 h after the initial injection. Data presented are averages plus standard deviations ($n = 3$ mice per group). The ages of LAT-Bam mice for this analysis were chosen based on the age at which these mice had similar spleen weights to 5- to 6-week-old and 12- to 13-week-old LAT-KI mice.

development, T cells from LAT-KI mice showed higher proliferative rates than those from LAT-Bam mice. However, we cannot distinguish a decrease in magnitude of proliferative rate from a kinetic delay in hyperproliferation in LAT-Bam mice. In separate adoptive transfer experiments, sorted LAT-KI CD4$^+$ T cells adoptively transferred into LAT knockout (T-cell–deficient) mice expanded more quickly than sorted LAT-Bam CD4$^+$ T cells (Fig. S4 and *SI Methods*). In conclusion, the collective evidence supports the hypothesis that LAT-KI T cells have a higher proliferative rate than LAT-Bam T cells.

We also previously reported that T cells from LAT-KI mice are resistant to TCR activation-induced cell death (AICD), which is another potential mechanism that could account for the large numbers of CD4$^+$ T cells observed in LAT-KI mice (8). To analyze the effect of Bam32 deficiency on AICD in LAT-Bam T cells, we performed a TCR restimulation assay on purified CD4$^+$ T cells from the various mutant mice (Fig. S5*A* and *SI Methods*). LAT-KI and LAT-Bam CD4$^+$ T cells showed a similar resistance to AICD. Therefore, differences in levels of AICD do not seem to explain the delay of lymphoproliferative disease seen in LAT-Bam compared to LAT-KI mice. Another mechanism that could potentially contribute to differences in degrees of lymphoproliferative disease in these mice is a lack of FoxP3$^+$ regulatory T cells, which has been previously reported for LAT-KI mice (11, 12). To address whether a pool of T-regulatory cells exists in LAT-Bam mice that might allow for a delay of lymphoproliferative disease in those mice, we assayed for the presence of FoxP3$^+$ T cells. FoxP3$^+$ T cells were nearly absent in LAT-Bam CD4$^+$ T cells as well as in LAT-KI CD4$^+$ T cells (Fig. S5*B*). In conclusion, of the mechanisms examined, a decrease in in vivo proliferative rate of LAT-Bam T cells appears to account for the delay in lymphoproliferative disease development seen in LAT-Bam mice.

To investigate what signaling pathways might have been altered by the Bam32 null mutation in LAT-KI T cells, we examined TCR-induced calcium flux and the activation status of MAP kinases in these mutant T cells. The LATY136F knock-in mutation disrupts PLC-γ1 binding and activation and severely reduces calcium flux after TCR stimulation of CD4$^+$ T cells (8). It is unlikely, but possible that a Bam32 null mutation would restore TCR-induced calcium influx in LAT-KI T cells. We measured calcium influx by flow cytometry under conditions of both strong and weak soluble antibody stimulation (Fig. S6 and *SI Methods*). Both LAT-KI and LAT-Bam CD4$^+$ T cells lacked calcium influx consequent to either weak or strong TCR stimulation.

We also analyzed the effect of the Bam32 null mutation on activation of the MAP kinases Erk, Jnk, and p38 since Bam32 knockout studies showed that Bam32 plays a role in activation of Erk and Jnk downstream from the BCR (23) and plays a role in Erk activation downstream of the TCR (25). We assayed Erk, Jnk, and p38 activation by measuring levels of phospho-Erk1 and 2, phospho-Jnk2, and phospho-p38 using western blotting of lysates from purified CD4$^+$ T cells. Since our previous studies showed a more prominent role for Bam32 in MAPK activation following suboptimal TCR stimulation, cells were stimulated through the TCR by two different methods. In the first method, we stimulated ex vivo purified CD4$^+$ T cells with platebound anti-CD3ε, which provides a low level of stimulation. In the second method, we rested purified CD4$^+$ T cells for 5 h in culture and then stimulated them with soluble higher concentration anti-CD3ε and anti-CD4 (coreceptor) cross-linking. As shown in Fig. 4, platebound antibody stimulation of ex vivo WT T cells resulted in minimal MAPK activation. However, *p*-Erk, *p*-Jnk, and *p*-p38 levels were much higher in LAT-KI than in WT CD4$^+$ T cells in the basal and stimulated states. Furthermore, Erk phosphorylation was decreased in LAT-Bam T cells relative to

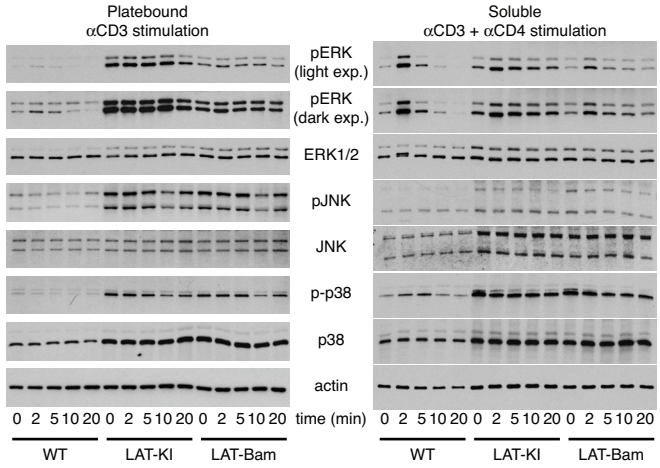

Fig. 4. MAPK activity in LAT-KI and LAT-Bam T cells. TCR-induced Erk, Jnk, and p38 phosphorylation were measured in mutant CD4$^+$ T cells by western blotting. For the left panel, purified CD4$^+$ lymph node T cells from mutant mice were stimulated with 5 μg/mL platebound anti-CD3ε for the indicated times at 37 °C. For the right panel, purified CD4$^+$ lymph node T cells were incubated for 5 h in culture and then were stimulated with 10 μg/mL soluble biotinylated anti-CD3ε and 10 μg/mL anti-CD4 and streptavidin cross-linking for the indicated times at 37 °C. Total cell lysates were analyzed by western blotting for the indicated proteins or phosphoproteins. Data are representative of at least two experiments.

LAT-KI T cells, although *p*-Jnk and *p*-p38 levels were similar. Although there was some mouse-to-mouse variation in *p*-Erk levels in LAT-Bam T cells, the levels were always intermediate between WT and LAT-KI levels. Soluble antibody stimulation of rested T cells also revealed basal activation of MAP kinases in LAT-KI compared to WT T cells, however activation through the TCR was better seen under these conditions. As with the platebound stimulation, LAT-Bam T cells had intermediate levels of Erk phosphorylation between WT and LAT-KI T cells, whereas *p*-Jnk and *p*-p38 levels were similar in LAT-Bam and LAT-KI T cells.

We were intrigued by the possibility that the diminution of Erk activation in LAT-Bam T cells relative to LAT-KI T cells might play a role in the delay of lymphoproliferative disease in the LAT-Bam mice, especially in light of the well-established link between Erk activity and proliferation in a variety of other systems (13). In addition, the loss of Bam32 might result in effects on any of a number of pathways. To more directly test the hypothesis that Erk activation specifically influences lymphoproliferative disease in LAT-KI mice, we interbred LAT-Bam mice with mice expressing a transgene that encodes a hypersensitive mutant of Erk2 driven from the human CD2 promoter (29). The Erk2 mutation is analogous to the sevenmaker mutation from *Drosophila* and is a hyperactivatable (not constitutively active) form of Erk2. Both the spleen weight and the number of CD4$^+$ T cells in the spleens of 7-week-old Erk transgenic, LAT-Bam mice were increased relative to LAT-Bam mice (Fig. 5). The surface phenotype and Th2-skewed nature of Erk transgenic LAT-Bam T cells were similar to LAT-KI and LAT-Bam T cells (Fig. S7). Infiltration of lymphocytes into lung, which is never observed in 7-week-old LAT-Bam mice, was observed in four of four 7-week-old Erk transgenic, LAT-Bam mice (Fig. 5). Finally, western blot analysis confirmed higher Erk phosphorylation levels in CD4$^+$ T cells from Erk transgenic, LAT-Bam mice than in CD4$^+$ T cells from LAT-Bam mice (Fig. S8). Therefore, of all of the potential changes resulting from the Bam32 null mutation in LAT-KI cells, we found that modulation of Erk activity is one that impacts lymphoproliferative disease.

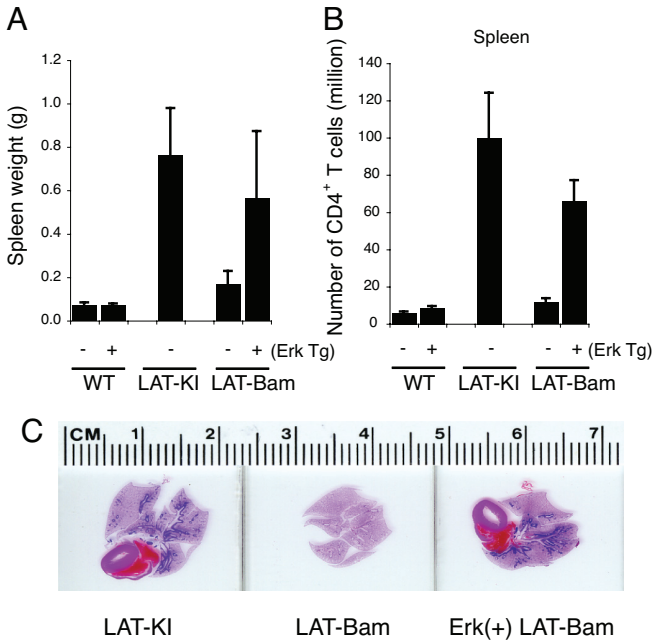

Fig. 5. T cell-driven expression of the Erk2 sevenmaker mutant in LAT-Bam mice exacerbates lymphoproliferative disease. (*A*) Averages of spleen weights for the indicated mice ($n = 3$) at 7 weeks of age are shown. (*B*) Numbers of peripheral CD4$^+$ T cells were determined by cell counting and flow cytometry for the indicated mice ($n = 3$) at 7 weeks of age. (*C*) Photomicrographs of hematoxylin and eosin-stained tissue sections of whole lungs from 7-week-old mice. LAT-KI and Erk transgenic LAT-Bam mice exhibit lymphoproliferative disease in the lungs, whereas LAT-Bam mice exhibit histologically unremarkable lungs at this age. The extent of lymphocyte infiltration in Erk transgenic, LAT-Bam lungs was variable (but detectable), and this section is an example of a more affected lung.

Discussion

We and others have previously described a mouse model in which the endogenous WT LAT gene is replaced by a mutant form of LAT (Y136F) that cannot recruit PLC-γ1 (8, 9). This mutation severely restricts T-cell development, and yet a subset of T cells accumulates with a memory or hyperactivated phenotype (8, 9). Two aspects of the phenotype of this mouse model system were of particular interest to us. First, a lymphoproliferative disease develops by the time the mice are 4–6 weeks of age and continues until their death, usually by 6 months of age. The lymphoproliferative disease is characterized by lymphadenopathy, splenomegaly, and multi-organ infiltration of lymphocytes and other immune cells. The proliferative T cells in LAT-KI mice are Th2-biased and infiltrate most readily into the lung. Second, when analyzing signal transduction in LAT mutant T cells, we discovered that TCR-induced calcium influx was abrogated, an expected downstream effect of the lack of activation of PLC-γ1. Another expected downstream effect of the lack of activation of PLC-γ1 is lack of DAG generation, which would be predicted to result in decreased RasGRP activity and consequently decreased MAPK activity. Surprisingly, however, MAPK activity was not decreased, and when analyzed here, was in fact increased in ex vivo LAT-KI T cells. Platebound stimulation through the TCR of ex vivo CD4$^+$ T cells best revealed differences in signaling between LAT-KI and WT CD4$^+$ T cells. Under these conditions, LAT-KI T cells exhibited higher levels of MAPK activation than WT T cells, especially in the basal (freshly isolated) state. We assume that these differences reflect the abnormal activation of MAP kinases in LAT-KI T cells in vivo, which could drive lymphoproliferative disease in LAT-KI mice.

We have been interested in determining whether genetic alterations in signaling adaptor molecules other that LAT can modulate lymphoproliferative disease in LAT-KI mice and MAPK activation in LAT-KI T cells. Because of the considerable increase in Erk activation in LAT-KI T cells, we are analyzing null mutants of molecules that are known to be involved in activation of the Ras/MEK/Erk pathway in T cells. For instance, we are interested in examining null mutants of RasGRP and Grb2 or Sos1 for the purpose of trying to discern the relative contributions of and the dynamic interplay between RasGRP and Grb2/Sos pathways in Erk activation in LAT-KI T cells. Some of these experiments are ongoing, however null mutants of Grb2 and Sos1 are embryonic lethal, therefore T-cell conditional knockouts must be generated and analyzed. In an effort to examine other molecules that might regulate Erk activation outside the classical RasGRP and Grb2/Sos pathways in LAT-KI T cells, we began to investigate another molecule, Bam32, that we showed could regulate Erk activity in T cells (25).

In this study, we report that Bam32 is a critical regulator of Erk phosphorylation and lymphoproliferative disease in LAT-KI T cells. LAT-Bam double-mutant mice developed a similar Th2-biased lymphoproliferative syndrome to LAT-KI mice, but the lymphoproliferative disease was delayed and reduced in severity. In addition to the difference in disease severity, a qualitative difference noted in the LAT-Bam mice was a near absence of pulmonary lymphocyte infiltration and proliferation, especially in younger (2- to 3-month-old) mice. Regarding MAPK activation, Erk activation was reduced in LAT-Bam T cells relative to LAT-KI T cells. We tested the significance of this reduction of Erk activation by introducing a hypersensitive allele of Erk2 into LAT-Bam mice. Expression of this Erk2 transgene in LAT-Bam T cells resulted in acceleration of splenomegaly and lymphadenopathy and the appearance of pulmonary lymphocyte infiltration in young (7-week-old) mice. Therefore, of all of the possible signaling alterations caused by the Bam32 null mutation, we observed a correlation between Erk activation and lymphoproliferative disease in LAT-Bam mice. Although we do not yet know the biochemical links between Bam32 and Erk, we are currently investigating these links based on the importance of Erk as a critical regulator of hyperproliferation in LAT-KI T cells and lymphoproliferative disease in LAT-KI mice.

LAT-KI, LAT-Bam, and Erk transgenic LAT-Bam T cells all produce IL-4 upon PMA and ionomycin stimulation in vitro and show hallmarks of Th2 skewing in vivo even though these mice have different rates of development of lymphoproliferative disease in vivo. Th2 skewing in LAT-KI mice may be caused by the disruption of calcium signaling since a pharmacological blockade of TCR-induced calcium signaling with calcineurin results in a shift toward a Th2 phenotype in vitro (30). In vivo, this Th2 bias, with overproduction of IL-4, can result in hyperactivation of B cells and autoantibody production in LAT-KI mice (10, 12). Although LAT-KI and LAT-Bam T cells show a Th2 bias, LAT-KI mice differ both quantitatively (earlier detection of IL-4 in serum) and qualitatively (more frequent pulmonary lymphocyte infiltration) from LAT-Bam mice. Although subtle differences in Th2 differentiation may exist between LAT-KI, LAT-Bam, and Erk transgenic LAT-Bam mice, the differences appear to be secondary to the numbers of peripheral CD4$^+$ T cells in these mouse models, which appear to be the primary determinant of extent of lymphoproliferative disease.

At least three mechanisms could contribute to the accumulation of the abundant number of CD4$^+$ T cells seen in LAT-KI mice. First, LAT-KI T cells have a higher in vivo proliferative rate than normal CD4$^+$ T cells (8) and in adoptive transfer

experiments, LAT-KI T cells can proliferate in an MHC-independent manner (12). Second, in in vitro experiments examining activation-induced cells death (AICD), LAT-KI T cells do not die as readily as WT $CD4^+$ T cells (8). Third, LAT-KI mice lack functional $FoxP3^+CD4^+CD25^+$ Treg cells (11, 12), which control lymphoid homeostasis (31). Of these three processes (T-cell proliferation, T-cell death, and Treg action), proliferation rate seems to be the major determinant of the difference in rate of development of lymphoproliferative disease between LAT-Bam and LAT-KI mice. All three of these processes are dependent on IL-2 production, which is deficient in LAT-KI mice (8, 9). Other mouse models in which calcium influx and IL-2 production are dampened or eliminated and lymphoproliferative disease results include STIM1/STIM2 knockouts (32), NFATc2/NFATc3 knockouts (33), and knockouts of IL-2 itself (34). In these mouse models, as well as in the LAT-KI model, although an initial expectation of these genetic alterations might be immune deficiency, lymphoproliferative disease develops instead. Lymphoproliferative disease in these models is due at least in part to a loss of IL-2-mediated AICD and IL-2-mediated Treg action. In an effort to better understand the development of lymphoproliferative disease in LAT-KI mice, we found that alteration of Erk activation, whether by introduction of the Bam32 null mutation in LAT-KI mice or by introduction of a hypersensitive Erk allele in LAT-Bam mice, influences the proliferation of LAT-KI T cells and the development of lymphoproliferative disease.

Materials and Methods

Mice. The LAT-Y136F knock-in mice and LAT knockout mice used in this study were described previously (4, 8). Bam32 mice (23) were provided by Michel Nussenzweig (Rockefeller University) and Erk2 transgenic mice (29) were provided by Stephen Hedrick [University of California, San Diego (UCSD)]. LAT-Bam mice were generated by mating homozygous or heterozygous LAT-KI and Bam32 KO mice and were genotyped by PCR analysis of mouse ear DNA. All mice used in this study were on a C57BL/6 background (LAT-KI were ≥N13, Bam32 KO were ≥N10, and Erk2 Tg were ≥N7) and were housed under specific pathogen-free conditions.

Flow Cytometry. Single-cell suspensions were analyzed by standard methods for flow cytometry using a FACSCalibur (BD Biosciences) and FlowJo analysis software (Tree Star).

In Vivo T Cell Proliferation. To assess BrdU incorporation of T cells in vivo, mice received 0.8 mg BrdU in PBS by i.p. injection at 0 and 6 h. At 24 h after the initial injection, single-cell suspensions were made from lymph nodes. Lymph node cells were stained with CD4-APC and anti-BrdU-FITC using the BrdU Flow kit (BD Biosciences). Percentages of $CD4^+$ T cells also positive for BrdU were determined by flow cytometry.

Western Blotting. $CD4^+$ T cells purified by positive magnetic bead purification (over 90% purity; Miltenyi Biotec) at room temperature were stimulated with 5 μg/mL platebound anti-CD3ε at 37 °C for the indicated times. Cells were lysed directly in SDS sample buffer containing β-mercaptoethanol and were sonicated. For gel electrophoresis, 10% Criterion gels (Bio-Rad Laboratories) were used. Total Erk and phospho-Erk levels were determined by blotting of total protein extracts with anti-Erk-1,2 (137F5) and anti-phospho-Erk-1,2 (D13.14.4E) antibodies (Cell Signaling Technology). For Jnk Western blotting, phospho-SAP/JNK (81E11) and JNK2 (56G8) antibodies were used (Cell Signaling Technology). For p38 western blotting, phospho-p38 (12F8) and p38 (rabbit polyclonal) antibodies were used (Cell Signaling Technology). For ECL detection, SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) was used.

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