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_J Immunol_ 2012; 189:4005-4013; Prepublished online 14 September 2012; doi: 10.4049/jimmunol.1201380

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The Importance of the Erk Pathway in the Development of Linker for Activation of T Cells-Mediated Autoimmunity

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The ability of the transmembrane adaptor protein linker for activation of T cells (LAT) to regulate T cell development, activation, survival, and homeostasis depends upon phosphorylation of its multiple tyrosine residues. The mutation of tyrosine 136 on LAT abrogates its interaction with phospholipase C-γ1, causing severe ramifications on TCR-mediated signaling. Mice harboring this mutation, LATY136F mice, have significantly impaired thymocyte development; however, they rapidly develop a fatal lymphoproliferative disease marked by the uncontrolled expansion of Th2-skewed CD4+ T cells, high levels of IgE and IgG1, and autoantibody production. In this study, we assessed the contribution of multiple signaling pathways in LATY136F disease development. The deletion of the critical signaling proteins Gads and RasGRP1 caused a further block in thymocyte development, but, over time, could not prevent CD4+ T cell hyperproliferation. Also, restoring signaling through the NF-κB and NFAT pathways was unable to halt the development of disease. However, expression of a constitutively active Raf transgene enhanced lymphoproliferation, indicating a role for the Ras–MAPK pathway in LAT-mediated disease.

Received for publication May 16, 2012. Accepted for publication August 20, 2012.

This work was supported by National Institutes of Health Grants AI048674 and AI056156.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Abbreviations used in this article: DN, double-negative; DP, double-positive; IKK, IkB kinase; LAT, linker for activation of T cells; PLC, phospholipase C; RasGRP1, Ras guanyl-releasing protein 1; SP, single-positive; Treg, T regulatory; WT, wild-type.

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system described above, mice containing LAT completely deleted in mature T cells (ERCε\textsuperscript{Cre}/LAT\textsuperscript{Δ2} mice) develop a similar autoimmune syndrome, although the disease observed is much less severe than the LATY136F disease (14). Together, the data from analyses of these LAT mutant mice clearly indicate the importance of this adaptor protein in T cell homeostasis.

In this study, we undertook the task of further illuminating the signaling components involved in LAT-mediated disease, using both LATY136F (LAT\textsuperscript{Δ2/m}) and ERCε\textsuperscript{Cre}/LAT\textsuperscript{Δ2/m} (LAT\textsuperscript{Δ2/m}) mice. We tested whether the deletion of the LAT signalosome components Gads, which interacts with LAT to recruit the essential cytoplasmic adaptor protein SLP-76, and RasGRP1, a critical Ras guanine exchange factor in T cells, would lessen disease severity. We subsequently examined whether restoring the signaling pathways that are disrupted upon the abrogation of the LAT–PLC-γ1 interaction, namely NFAT, NF-κB, and Erk activation, could ameliorate disease development. Our data indicated that the Ras–MAPK pathway plays an important role in the development of LAT-mediated autoimmunity.

**Materials and Methods**

**Mice**

For all experiments, age-matched LAT\textsuperscript{Δ2/m} or ERCε\textsuperscript{Cre}/LAT\textsuperscript{Δ2/m} mice were used as controls and are referred to as WT, ERCε\textsuperscript{Cre}/LAT\textsuperscript{Δ2/m} and ERCε\textsuperscript{Cre}/LAT\textsuperscript{Δ2/m} mice were generated, as described previously (13, 15). To generate transgenic mice expressing constitutively active calcineurin, cDNA encoding a mutant form of the calcineurin catalytic subunit (16), in which the calmodulin binding domain and autoinhibitory domain were deleted, was cloned into the p29Δ2(Sal') vector. Ikk2α mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Ralβ mice were provided by R. Perlmutter (University of Washington, Seattle, WA). Gads\textsuperscript{-/-} mice were from A. Cheng (Washington University, St Louis, MO), and RasGRP1\textsuperscript{-/-} mice were obtained from J. Stone (University of Alberta, Edmonton, AB, Canada). Mice were housed in specific pathogen-free conditions and were used in accordance with National Institutes of Health guidelines. The experiments described in this study were reviewed and approved by the Duke University Institutional Animal Care and Use Committee.

**In vivo deletion of LAT**

Tamoxifen (Sigma-Aldrich) was dissolved in corn oil to the final concentration of 10 mg/ml. Eight- to 10-wk-old mice were injected i.p. with 200 μl tamoxifen on two consecutive days, followed by injection once per week for 4–8 wk. In all instances, LAT deletion, as determined by GFP expression, was >98% in CD4 T cells.

**Flow cytometry analysis and Abs**

Fluorescent Abs used in flow cytometry, such as anti-CD4, anti-CD8, anti-CD62L, anti-CD44, and anti-TCRβ, were all purchased from BioLegend. For surface marker staining, single-cell suspensions were prepared from thymuses, spleens, or lymph nodes and were blocked with anti-FcγRII/III before staining with different Ab mixtures. For intracellular staining of Foxp3, cells were fixed after surface marker staining and permeabilized, according to the manufacturer’s instructions (BD Biosciences). Cells were then stained on ice in the dark with anti-Foxp3 Ab. All FACS data were acquired on FACSCanto (BD Biosciences) and analyzed with FlowJo software.

**Intracellular cytokine production and staining**

For intracellular staining of cytokines, cells were isolated and stimulated with PMA (20 ng/ml) and ionomycin (0.5 μg/ml) in RPMI 1640 complete for 1 h before the addition of monensin. Cells were left for an additional 3 h before being stained for cell surface markers and then fixed and permeabilized, per the manufacturer’s instructions (eBioscience). Samples were then stained with intracellular Abs, such as anti–IFN-γ, anti–IL-2, and anti–IL-4 Abs (eBioscience). Flow cytometry data were acquired on FACSCanto (BD Biosciences) and analyzed with FlowJo software.

**Results**

**LAT-mediated disease development in the absence of Gads**

LAT\textsuperscript{Δ2/m} thymocytes undergo a partial block in development, with a small number of T cells escaping into the periphery to instigate disease (10, 11). Because the Gads-SLP-76 complex is still able to bind to LAT upon the mutation of tyrosine 136 (4), activation of PLC-γ1 by the prolinc-rich P1 domain of SLP-76 may still occur in LAT\textsuperscript{Δ2/m} T cells (17, 18). Therefore, we hypothesized that LAT-mediated phosphorylation of PLC-γ1 through Gads-SLP-76 could be driving the residual thymocyte development and T cell proliferation. We thus investigated the role of the Gads-SLP-76 complex in the development of LATY136F autoimmunity.

To assess the contribution of Gads to disease development, we crossed Gads\textsuperscript{-/-} with LAT\textsuperscript{Δ2/m} to generate Gads\textsuperscript{-/-}/LAT\textsuperscript{Δ2/m} mice. As shown in Fig. 1A, total numbers of thymocytes were reduced in LAT\textsuperscript{Δ2/m}, Gads\textsuperscript{-/-}, and Gads\textsuperscript{-/-}/LAT\textsuperscript{Δ2/m} mice. LAT\textsuperscript{Δ2/m} thymocytes had a partial block in development, with a small percentage of cells escaping the DN compartment and maturing to the DP stage (Fig. 1B). The deletion of Gads also led to a marked defect in thymocyte development, with an increased percentage of DN cells and reduced DP and SP compartments compared with WT mice, as published previously (6). In contrast, LAT\textsuperscript{Δ2/m}Gads\textsuperscript{-/-} thymocytes had a near-complete block at the DN stage of thymocyte development and contained virtually no DP or SP cells. By 1 mo of age, LAT\textsuperscript{Δ2/m} mice had splenomegaly, and the number of CD4+ T cells in these mice was greatly increased (Fig. 1A). Despite a partial block in thymocyte development, LAT\textsuperscript{Δ2/m} CD4+ T cells had undergone expansion to account for 23% of splenocytes (Fig. 1C). Gads\textsuperscript{-/-} mice were T cell lymphopenic, as spleens were 4% CD8+ and ∼2% CD4+ T cells. However, due to the severe thymocyte block, LAT\textsuperscript{Δ2/m}Gads\textsuperscript{-/-} spleens contained <1% of both CD8+ and CD4+ cells. These data suggested that, in the absence of the LAT–PLC-γ1 interaction, Gads is critical for the emanation of signals in developing T cells.

To bypass the severe thymocyte developmental block observed in LAT\textsuperscript{Δ2/m}Gads\textsuperscript{-/-} mice, we also used ERCε\textsuperscript{Cre}/LAT\textsuperscript{Δ2/m} Gads\textsuperscript{-/-} mice to further analyze the role of Gads in LAT-mediated autoimmune immunity. ERCε\textsuperscript{Cre}/LAT\textsuperscript{Δ2/m}, ERCε\textsuperscript{Cre}/LAT\textsuperscript{Δ2/m}, and ERCε\textsuperscript{Cre}/LAT\textsuperscript{Δ2/m} Gads\textsuperscript{-/-} were used as controls. Because mice with the floxed LAT allele used in this study also carry the ERCε transgene, we omitted ERCε from the description of mouse genotypes below for simplicity. These mice were all treated with tamoxifen for 8 wk to delete the WT LAT allele. The deletion of LAT led to splenomegaly and hyperproliferation of CD4+ T cells in LAT\textsuperscript{Δ2/m} mice (Fig. 2A), as described previously (13). LAT\textsuperscript{Δ2/m}Gads\textsuperscript{-/-} spleens were slightly smaller than LAT\textsuperscript{Δ2/m} spleens, but LAT\textsuperscript{Δ2/m}Gads\textsuperscript{-/-} mice displayed severe splenomegaly, with only slightly reduced spleen weights. LAT\textsuperscript{Δ2/m} spleens contained large percentages of CD4+ cells (Fig. 2B). LAT\textsuperscript{Δ2/m}Gads\textsuperscript{-/-} spleens, in contrast, had reduced percentages of CD4+ and CD8+ T cells. LAT\textsuperscript{Δ2/m}Gads\textsuperscript{-/-} spleens contained ~25% CD4+ T cells and, like LAT\textsuperscript{Δ2/m} spleens, had virtually no CD8+ T cells. Also, the vast majority of CD4+ T cells in LAT\textsuperscript{Δ2/m}, LAT\textsuperscript{Δ2/m}Gads\textsuperscript{-/-}, and LAT\textsuperscript{Δ2/m}Gads\textsuperscript{-/-} spleens bore an effector/memory phenotype of CD44\textsuperscript{hi}CD62L\textsuperscript{lo}. The CD4+ T cells in LAT\textsuperscript{Δ2/m}Gads\textsuperscript{-/-} mice expressed lower levels of surface TCR than WT cells, and LAT\textsuperscript{Δ2/m}Gads\textsuperscript{-/-} CD4+ TCR levels were even further decreased compared with LAT\textsuperscript{Δ2/m} T cells (Fig. 2C). These data indicated that Gads is not required for the development of LATY136F-mediated autoimmunity.

One of the hallmark characteristics of the LATY136F disease is the heightened production of Th2 cytokines, namely IL-4 (10, 11). Thus, we wanted to assess the cytokine production by LAT\textsuperscript{Δ2/m} Gads\textsuperscript{-/-} and LAT\textsuperscript{Δ2/m}Gads\textsuperscript{-/-} T cells (Fig. 2D). A small percentage of WT cells produced IFN-γ upon stimulation. In contrast to LAT\textsuperscript{Δ2/m} cells, which produce solely IL-4, ~30% of LAT\textsuperscript{Δ2/m} T cells produced IFN-γ and ~10% of LAT\textsuperscript{Δ2/m} cells produced both cytokines. Furthermore, an increased percentage of LAT\textsuperscript{Δ2/m}Gads\textsuperscript{-/-} CD4+ T cells stained positive for IFN-γ and IL-4 compared with WT cells. Noticeably, LAT\textsuperscript{Δ2/m}Gads\textsuperscript{-/-} T cells...
LATf/m, LATf/+,
LATm/m, LATm/+,
LATm/mRasGRP1

1. Severely impaired thymocyte development in LATm/mGads−/− mice. (A) Total numbers of thymocytes, splenocytes, and CD4+ T cells in WT, LATm/m, Gads−/−, and LATm/mGads−/− mice. (B) Representative FACS plots of CD4 versus CD8 expression on thymocytes from 4-wk-old mice. (C) Surface expression of CD4 and CD8 on splenocytes from 4-wk-old mice. Data are representative of three independent experiments using two to four mice per genotype.

Ablation of RasGRP1 does not inhibit disease development

Because the deletion of Gads in LATf/m T cells was unable to prevent the development of disease, we next evaluated whether disruption of TCR-mediated Ras/Erk signaling through the deletion of RasGRP1 would be able to inhibit CD4+ T cell proliferation in LATm/m mice. RasGRP1, a Ras guanine exchange factor, serves to activate Ras following PLC-γ1 activation and diacylglycerol production (7). The deletion of RasGRP1 in T cells leads to impaired thymocyte development and Erk activation (9). Because RasGRP1 plays such a critical role in mediating Ras/Erk activation in T cells, we hypothesized that its deletion in LATm/m T cells could stymie disease development. To this end, we crossed LATm/m with RasGRP1−/− to generate LATm/mRasGRP1−/− mice.

As seen previously, LATm/m thymocytes were partially blocked during the DN stage of development (Fig. 3A). RasGRP1−/− mice had increased DN and DP compartments, with very few cells progressing to either the CD4 or CD8 SP stage. Analysis of LATm/m RasGRP1−/− mice revealed that thymocytes had a more severe block in the DN stage than LATm/m mice, with only 7.4% of thymocytes becoming DP cells compared with 26.6% of LATm/m thymocytes. By 1 mo of age, LATm/m spleens already contained a significant percentage of CD4+ T cells (Fig. 3B). In contrast, RasGRP1−/− spleens had severely reduced CD8+ and CD4+ populations. And, as a result of the impaired thymic development, LATm/mRasGRP1−/− mice contained negligible amounts of splenic T cells.

To bypass the development block and more definitively analyze the role of RasGRP1 in LAT-mediated disease, we also employed LATm/m mice. LATm/m, LATm/+,
LATm/mRasGRP1−/−, and LATm/m RasGRP1−/− mice were treated with tamoxifen for 8 wk. Expression of the mutant LAT allele resulted in splenomegaly and increased spleen cell numbers (Fig. 4A). LATm/mRasGRP1−/− spleens were comparable, if slightly larger than LATm/+ spleens. Notably, LATm/mRasGRP1−/− spleens were similar in size and splenocyte number to those of the mutant mice. LATm/m and LATm/m RasGRP1−/− mice contained similarly high percentages of CD4+ T cells, all of which bore an effector/memory phenotype (Fig. 4B). In addition, only the CD4+ T cells from LATm/mRasGRP1−/− mice produced IL-4 (Fig. 4C). These data suggested a minimal role of RasGRP1 in LAT-mediated disease.

We further analyzed aged LATm/mRasGRP1−/− mice to determine whether the few T cells found in young mice could instigate disease over time. By 4 mo of age, LATm/mRasGRP1−/− mice developed a disease similar to that in LATm/m mice, although severity varied in individual mice (Fig. 5A). Whereas some mice had moderately increased spleen weights and cellularity compared with WT mice, other littersmates had striking splenomegaly, similar to that of age-matched LATm/m mice. By this age, LATm/m spleens were ~50–80% CD4+ T cells and contained virtually no CD8+ T cells (Fig. 5B). RasGRP1−/− spleens contained a similar percentage of CD4+ T cells compared with WT spleens, but a much-reduced CD8+ compartment. LATm/mRasGRP1−/− mice all had increased, but variable, percentages of CD4+ cells, from ~23 to 70%. Again, none of these mice contained significant numbers of CD8+ cells. Additionally, almost all CD4+ T cells in LATm/m, RasGRP1−/−, and LATm/mRasGRP1−/− mice were CD62Llow and CD44high. Similar to LATm/m cells, LATm/mRasGRP1−/− CD4+ T cells produced tremendous amounts of IL-4 (data not shown). From these data, we were able to conclude that the deletion of RasGRP1 significantly impaired thymocyte development, but over time was unable to inhibit the uncontrolled proliferation of mutant T cells.

We also assessed whether the deletion of RasGRP1 and Gads could affect T regulatory (Treg) cell populations. As we previously reported (19), Foxp3+ cells were nearly absent in LATm/m mice (Fig. 5C). RasGRP1−/− mice had similar or even slightly increased percentages of Foxp3+ cells compared with WT controls. Foxp3+ T cells were clearly unable to develop in LATm/m RasGRP1−/− mice. Because 4-mo-old LATm/mRasGRP1−/− mice developed the disease, we analyzed LATm/mGads−/− mice of a similar age. LATm/mGads−/− mice also developed a similar disease as LATm/m mice (data not shown). Despite a severe block in thymocyte development (Fig. 1), there was a significant pop-
ulation of CD4+ T cells (11.7%) in 4-mo-old LATm/mGads−/− mice, most likely due to tremendous expansion of these cells (Fig. 5D). We also assessed whether Gads deficiency had any impact on the development of Treg cells. Despite having decreased CD4+ T cell percentages, Gads−/− spleens actually contained a similar percentage of Foxp3+ T cells in the CD4+ compartment compared with WT controls. However, LATm/mGads−/− mice, similar to LATm/m mice, almost completely lacked Foxp3+ T cells. These data indicated that, whereas RasGRP1 and Gads are critical for conventional T cell development, they are not required for the development of Treg cells.

Expression of constitutively active calcineurin does not rescue LAT-mediated disease

Because disease development was not impeded by the deletion of Gads or RasGRP1, we next determined whether reconstituting the signaling pathways downstream of the LAT–PLC-γ1 interaction could help restore proper T cell function. We first assessed the role of calcineurin signaling in LAT-mediated disease, as perturbation of the LAT–PLC-γ1 interaction causes LATm/m T cells to have a severe defect in calcium mobilization and NFAT activation (10). Calcineurin is a calcium/calmodulin-dependent phosphatase whose activity is required for the translocation of NFAT to the nucleus and, ultimately, T cell cytokine production and proliferation (20). Thus, we wanted to assess whether the expression of constitutively active calcineurin could restore proper T cell development and activation in the LAT-mediated disease.

To test whether the enforced expression of calcineurin could ameliorate disease development, we generated transgenic mice expressing constitutively active calcineurin under the human CD2 promoter. These mice (CnA+) contained a form of calcineurin in which the calmodulin binding domain and autoinhibitory domain were deleted. These mice were then crossed with LATm/m mice to produce LATm/mCnA+ mice. As previously reported, expression of constitutively active calcineurin impairs thymocyte development (16). Therefore, LATm/mCnA+ mice did not develop disease by 3
mo of age due to a severe block in thymocyte development that prevented T cells from populating the periphery in these mice (data not shown). We crossed mice expressing constitutively active calcineurin onto the LATf/m background to eliminate this developmental defect.

LATf/mCnA+ mice developed disease after 4 wk of tamoxifen treatment comparably to LATf/m mice. Like LATf/m mice, they had splenomegaly and lymphadenopathy (data not shown). Analysis of lymph nodes showed that WT mice had normal percentages of CD4+ and CD8+ cells, whereas LATf/m mice contained only CD4+ T cells (Fig. 6A). CnA+ mice had reduced percentages of peripheral T cells, although the CD4:CD8 ratio was relatively normal. LATf/mCnA+ mice contained predominantly CD4+ T cells, indicating that restoring NFAT signaling did not prevent the hyperproliferation of mutant T cells. In addition, CD4+CD25+ Treg cells were absent in these mice (data not shown). Together, these

FIGURE 3. Thymocyte development is impaired in LATm/mRasGRP1−/− mice. (A) The numbers of thymocytes, total splenocytes, and CD4+ T cells in WT, LATm/m, GRP1−/−, and LATm/mGRP1−/− mice. (B and C) FACS plots of CD4 and CD8 expression on live thymocytes (B) and splenocytes (C) from 4-wk-old mice. Data are representative of four independent experiments analyzing at least two mice per genotype.

FIGURE 4. Deletion of RasGRP1 does not inhibit disease development. Mice were treated with tamoxifen to delete the WT LAT allele for 8 wk before analysis. (A) Spleen weights and the numbers of splenocytes and CD4+ T cells from mice of the genotypes indicated. (B) CD4 versus CD8 FACS profiles of splenocytes. (C) Cytokine production. Splenocytes were stimulated with PMA and ionomycin in the presence of monensin for 4 h before intracellular staining. Data are representative of three independent experiments using two to five mice per genotype.
data provided sufficient evidence that restoring NFAT signaling did not prevent disease development.

NF-κB activation does not rescue LAT-mediated autoimmunity

Whereas enforced activation of calcineurin did not lessen disease severity, we also examined the role of another critical pathway downstream of the LAT–PLC-γ1 interaction, canonical NF-κB signaling. The NF-κB family members are normally sequestered in the cytoplasm through association with the inhibitory IκB family. Signaling through the TCR, among other stimuli, induces the IκB kinase (IKK) complex to phosphorylate IκB, which is then ubiquitinated and degraded, allowing for nuclear translocation of NF-κB (21). The IKK complex consists of two kinases, IKK1/a and IKK2/β, as well as a regulatory subunit, NF-κB essential modulator/IKKg. We generated LATf/mIKK2ca (constitutively active IKKb) mice by crossing LATf/m mice to a strain generated by Sasaki et al. (22), in which a constitutively active form of IKK2 preceded by a loxP-flanked STOP cassette is inserted into

FIGURE 5. RasGRP1 in LAT-mediated autoimmune disease. (A) Spleen weights and the numbers of total splenocytes and CD4+ cells from 4-mo-old mice of the indicated genotypes. (B) Representative FACS plots of CD4 and CD8 expression on splenocytes (top) as well as CD44 versus CD62L expression on CD4+ splenocytes (bottom). (C) and (D) Foxp3 expression in CD4+ T cells by intracellular staining. Data are representative of three independent experiments using two to three mice per genotype.

FIGURE 6. Restoration of NFAT and NF-κB signaling cannot suppress the LAT-mediated disease. Mice were treated with tamoxifen for 4 wk before analysis. (A) Representative FACS plots of CD4 versus CD8 expression on lymph node cells. (B) Surface expression of CD4 and CD8 on lymph node cells. The data shown are representative of two independent experiments using one to two mice per genotype.
the ROSA26 locus. In the presence of Cre, the floxed stop codon is excised, allowing for the expression of constitutively active IKK2, as well as the bicistronic expression of EGFP.

Mice were injected with tamoxifen for 4 wk to induce both the deletion of the floxed WT LAT allele and the expression of active IKK2. Analysis of thymuses showed that the expression of constitutively active IKK2 did not visibly affect thymocyte development and did not rescue thymocyte development in LAT<sup>fl/fl</sup> mice (data not shown). Analysis of disease development clearly showed that expression of constitutively active IKK2 did nothing to ameliorate splenomegaly, as LAT<sup>fl/fl</sup>IKK2ca spleens were similar in size to LAT<sup>fl/fl</sup> spleens (data not shown). In the periphery, IKK2ca mice contained similar percentages of CD4<sup>+</sup> and CD8<sup>+</sup> T cells compared with WT mice. LAT<sup>fl/m</sup> and LAT<sup>fl/m</sup>IKK2ca lymph nodes consisted of very few CD8<sup>+</sup> cells and large percentages of CD4<sup>+</sup> T cells, most of which expressed an effector-memory phenotype. T<sub>reg</sub> cells were also absent in these mice (Fig. 6B and data not shown). In sum, our data demonstrated that T cell hyperproliferation cannot be corrected by restoration of NFAT or NF-κB signaling.

**Constitutive activation of Raf augments LATY136F disease**

As the activation of the Ras/Raf/MEK/Erk signaling cascade is a crucial component of TCR-mediated signaling downstream of PLC-γ1 activation, we decided to further investigate the role of this pathway in LAT-mediated autoimmunity. We used transgenic mice expressing a constitutively active form of Raf, which harbors a CAAX farnesylation sequence at its C terminus. The farnesylation sequence directs Raf to cell membranes, imitating Ras-mediated Raf activation, thus causing constitutive activation of Raf/MEK/Erk (23).

We crossed constitutively active Raf (Raf<sup>Tg</sup>) mice with LAT<sup>fl/m</sup> mice to generate LAT<sup>fl/m</sup>Raf<sup>Tg</sup> mice. The size of thymi from LAT<sup>fl/m</sup>Raf<sup>Tg</sup> mice was similar to that of LAT<sup>fl/m</sup> mice, but the numbers of thymocytes in these mice were drastically reduced (Fig. 7A). Expression of Raf-CAAX in WT mice had no significant effect on thymocyte development (data not shown). Although there are very few DP and SP thymocytes in LAT<sup>fl/m</sup> mice, we observed virtually no DP cells and ~70% CD4<sup>+</sup> cells in LAT<sup>fl/m</sup>Raf<sup>Tg</sup> thymuses (Fig. 7A). It was shown previously that Raf-CAAX was able to drive RAG2<sup>−/−</sup> thymocytes to develop from DN to DP (24). Thus, it may accelerate LAT<sup>fl/m</sup> thymocyte differentiation from DP to SP. It is also possible that an increased percentage of CD4<sup>+</sup> T cells is a consequence of extensive expansion of LAT<sup>fl/m</sup>Raf<sup>Tg</sup> T cells in the thymus. Furthermore, spleens and lymph nodes from LAT<sup>fl/m</sup>Raf<sup>Tg</sup> mice were tremendously enlarged and splenocyte numbers were greatly increased compared with LAT<sup>fl/m</sup> mice (Fig. 7B). LAT<sup>fl/m</sup>Raf<sup>Tg</sup> spleens consisted of incredibly high percentages of CD4<sup>+</sup> T cells, similar to and even exceeding the percentage of CD4<sup>+</sup> T cells seen in LAT<sup>fl/m</sup> spleens (Fig. 7C). Expression of Raf-CAAX failed to correct the developmental defect of T<sub>reg</sub> cells in LAT<sup>fl/m</sup> mice, as no Foxp3<sup>+</sup> T cells were detected by intracellular staining in LAT<sup>fl/m</sup> and LAT<sup>fl/m</sup>Raf<sup>Tg</sup> mice (data not shown). Interestingly, there was a drastic reduction in the number of B cells in spleens and lymph nodes from LAT<sup>fl/m</sup>Raf<sup>Tg</sup> mice, especially from aged mice.

**FIGURE 7.** Constitutively active Raf augments disease severity. (A) The number of thymocytes and FACS plots on thymocytes. (B) Spleens and lymph nodes from mice of the indicated genotype (left) and total splenocyte numbers (right). (C) CD4 versus CD8 expression on splenocytes. (D) Forward scatter versus side scatter analysis of eosinophil populations. The data shown are representative of three independent experiments using one to two mice per genotype.
Whereas B cell development in LATm/m mice was largely normal, it was partially blocked in LATm/m/RafTg mice (data not shown). Another mark of the LATY136F disease is severe eosinophilia in peripheral lymphoid organs (10), as seen in Fig. 7D. LATm/m/RafTg lymph nodes had a higher percentage of eosinophils as compared with control and even LATm/m lymph nodes, indicating an aggressive disease. These data demonstrated that the transgenic expression of Raf in the context of the LATY136F mutation actually exacerbated disease development, implying an important role for the Ras/Erk pathway in guiding disease development mediated by the LAT mutation.

Discussion

From the data gathered in this study, it was obvious that the absence of Gads or RasGRP1 had a significant effect on LATm/m thymocyte development. Normally, LATm/m thymocytes exhibit an incomplete block in the DN3 stage (10, 11). In these mice, the DP population constitutes ∼20–30% of thymocytes, and SP compartments are reduced, yet distinct. LATm/m/Gads−/− mice displayed a near-complete block in the DN stage of thymocyte development, with virtually no DP or SP compartments. LATm/m RasGRP1−/− mice also had a more severe block than LATm/m mice, with only ∼7% of thymocytes progressing to the DP stage. Because LAT retains partial function upon mutation of tyrosine 136, a number of signaling pathways remain intact following the loss of the LAT–PLC-γ1 interaction and allow for a minimal amount of thymocyte differentiation. These pathways seem to act predominantly through Gads, as the ablation of this protein severely hindered thymocyte development. As the deletion of RasGRP1 also increased the developmental block of mutant thymocytes, one or more of these signaling pathways might also use RasGRP1. Perhaps the deletion of these proteins diminished or abolished the residual calcium flux and/or MAPK activation in LATm/m thymocytes. Interestingly, despite having virtually no peripheral T cells by ∼1 mo of age, LATm/m/RasGRP1−/− mice developed disease comparably to LATm/m mice by 4 mo of age, indicating that the few T cells able to populate the periphery were sufficient to instigate autoimmune.

Despite the dramatic effect on thymocyte development, LAT-mediated disease as driven by mature T cells in the LATm/m model was not significantly affected by the loss of Gads or RasGRP1. A previous report demonstrated that the uncontrolled hyperproliferation and Th2 effector function of LATY136F T cells are not dependent on MHC class II (25). However, another study examining the disease mediated by mature mutant T cells demonstrated that LATmY136F cells, which are similar to our ERCre+ LATm/m T cells, require MHC class II to initiate disease upon WT LAT deletion (26). These data suggest that LAT-mediated disease onset may require TCR engagement, whereas the perpetuation of the disease may be TCR independent. Thus, it seemed probable that continuous tonic signaling through the TCR could be driving the hyperproliferation of mutant T cells. Through the disruption of Gads and RasGRP1, we were able to effectively disrupt TCR-proximal signaling and observe the effect on mutant T cells. Both LATm/m/Gads−/− and LATm/m/RasGRP1−/− CD4+ T cells had decreased surface expression of TCRβ compared with LATm/m cells. Yet, LATm/m/Gads−/− and LATm/m/RasGRP1−/− mice developed disease similarly to LATm/m mice following 8 wk of tamoxifen treatment, suggesting that tonic signaling through the TCR is not driving the lymphoproliferative disease.

In addition to the impaired central tolerance seen in LATm/m mice, results from our laboratory show that these mice fail to develop peripheral tolerance and indeed lack natural CD4+CD25+ T regulatory cells (19). Adoptive transfer of WT Treg cells into neonatal LATm/m mice prevents the development of the autoimmune disease, highlighting the importance of natural Treg cells in the suppression of the LATY136F autoimmune syndrome. Studies have demonstrated that c-Rel, a member of the NF-κB family, is needed for thymic Treg differentiation and regulates Foxp3 transcription by directly binding to cis-regulatory elements at the Foxp3 locus (27, 28). Additionally, NFAT may bind to the foxp3 promoter and play a role in TCR-induced Foxp3 expression (29). However, restoration of these signaling pathways in LATm/m mice did not rescue Treg cell development and failed to prevent disease progression, suggesting that defects in these pathways are not the main reason causing the disease in these mice.

From the data detailed in this report, it is apparent that the LAT-mediated autoimmune disease was able to occur independently of RasGRP1 and Gads, two important molecules in TCR-proximal signaling. A previous study reported that decreasing Erk activation through the deletion of Bam32, an adaptor protein known to activate MAPK in B cells, lessens the severity of LATY136F disease (30). In contrast to this, the deletion of RasGRP1, despite increasing the thymocyte developmental block, did not impede progression of disease over time. Perhaps, in the context of this disease, the Grb2-Sos complex, which is able to bind directly to the phosphorylated TCR ζ-chain to activate Ras (31), may be able to compensate for the RasGRP1 deficiency and drive T cell expansion. Notably, expression of constitutively active Raf increased disease severity, as indicated by the tremendous spleen size, CD4+ proliferation, and eosinophilia observed in LATm/m/RafTg mice. These data support the idea that the Ras/MAPK pathway plays a significant role in guiding LATm/m T cell hyperproliferation. In this context, Raf may serve to enhance positive selection of mutant thymocytes, as Erk activation is known to contribute to positive selection (32, 33). Also, the presence of Raf-CAAX most likely led to persistent Erk activation, causing increased proliferation of mutant T cells. Additionally, if T cell proliferation in LATm/m mice is driven by cytokines, it is possible that Raf-CAAX enhanced Ras→MAPK activation downstream of cytokine receptor signaling and, consequently, increased LATm/m T cell proliferation.

In sum, we have shown that deletion of critical TCR signaling components, Gads and RasGRP1, did not ameliorate the LAT-dependent lymphoproliferative syndrome. Moreover, the restoration of signaling pathways immediately downstream of LAT could not rescue the development of the LATY136F disease. In fact, by enhancing MAPK signaling through the expression of constitutively active Raf, we significantly increased the severity of the disease, indicating that the Erk pathway does indeed contribute to the uncontrolled T cell expansion that occurs upon abrogation of the LAT→PLC-γ1 interaction.

Acknowledgments

We thank the Duke University Cancer Center flow cytometry, DNA sequencing, and transgenic mouse facilities for excellent services.

Disclosures

The authors have no financial conflicts of interest.

References


