LAT-mediated signaling in CD4$^+$CD25$^+$ regulatory T cell development

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Engagement of the T cell receptor for antigen (TCR) induces formation of signaling complexes mediated through the transmembrane adaptor protein, the linker for activation of T cells (LAT). LAT plays an important role in T cell development, activation, and homeostasis. A knock-in mutation at Tyr136, which is the phospholipase C (PLC)–γ1–binding site in LAT, leads to a severe autoimmune disease in mice. In this study, we show that CD4$^+$CD25$^+$ T reg cells that expressed Foxp3 transcription factor were nearly absent in both thymus and peripheral lymphoid organs of LAT^{Y136F} mice. This defect was not a result of the autoimmune environment as LAT^{Y136F} T reg cells also failed to develop in healthy LAT$^{-/-}$ mice that received mixed wild-type and LAT^{Y136F} bone marrow cells. Moreover, adoptive transfer of normal CD4$^+$CD25$^+$ T reg cells protected neonatal LAT^{Y136F} mice from developing this disease. These T reg cells effectively controlled expansion of CD4$^+$ T cells in LAT^{Y136F} mice likely via granzymes and/or TGF-β–mediated suppression. Furthermore, ectopic expression of Foxp3 conferred a suppressive function in LAT^{Y136F} T cells. Our data indicate that the LAT–PLC-γ1 interaction plays a critical role in Foxp3 expression and the development of CD4$^+$CD25$^+$ T reg cells.

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Abbreviations used: GFP, green fluorescence protein; GITR, glucocorticoid-induced TNF receptor; LAT, linker for activation of T cells; PLC, phospholipase C.

Regulatory T (T reg) cells are subsets of CD4$^+$ T cells that play crucial roles in immunological tolerance, transplantation, and autoimmunity (1–3). Among different types of T reg cells, naturally arising CD4$^+$CD25$^+$ T reg cells are the best characterized and studied. These cells comprise ~5–10% of CD4$^+$ T cells in peripheral lymphoid organs and represent a unique T cell lineage that undergoes thymic selection and migrates to the periphery (4–8). Natural T reg cells are characterized by cell surface expression of CD25 (the IL-2 receptor α-chain), CD62L (L-selectin), cytotoxic T lymphocyte antigen 4, and glucocorticoid-induced TNF receptor (GITR). The role of T reg cells in control of autoimmune diseases was clearly demonstrated in the experimental models in which elimination of CD25$^+$ T cells results in acceleration of disease progression including inflammatory bowel disease, experimental autoimmune encephalomyelitis, and autoimmune diabetes (9).

Multiple signaling pathways govern the development, function, and homeostasis of natural T reg cells including the IL-2 (10–15) and CD28 signaling pathways (16–18). In addition, the forkhead transcription factor, Foxp3, has recently been shown to be an essential regulator of CD4$^+$CD25$^+$ T reg cell development (19–21). Studies using Foxp3 knock-out mice (*Foxp3$^{-/-}$*) have shown that ablation of Foxp3 expression results in mice lacking CD4$^+$CD25$^+$ T reg cells and the development of the lethal lymphoproliferative autoimmune disease characterized by the uncontrolled proliferation of activated T cells. This disease can be prevented by transfer of CD4$^+$CD25$^+$ T reg cells. Moreover, overexpression of Foxp3 in CD4$^+$CD25$^-$ T cells converts these lymphocytes into CD4$^+$CD25$^+$ T cells with suppressive biological function (21). These findings suggest an obligatory role of Foxp3 in the development of natural T reg cells.

Engagement via the TCR is required for CD4$^+$CD25$^+$ T cell development (22–26). TCR engagement activates tyrosine kinases that further phosphorylate many cellular proteins, leading to activation of signaling events such as Ras–MAPK activation and calcium flux. A key protein that couples the signaling events to TCR engagement is the linker for activation of T cells (LAT) (27). LAT is a transmembrane adaptor protein that binds Grb2, Gads, and PLC-γ1. It is essential during T cell activation and thymocyte development (28). In addition, LAT also plays an important role in

immune homeostasis. Mice with a knock-in mutation of LAT at the PLC-γ1 binding site (Y136) show a severe autoimmune disease (29, 30). Although TCR-mediated phosphorylation of LAT and PLC-γ1 and Ca2^{+} mobilization in LATY136F T cells are markedly reduced, T cells in these mice are hyperactivated and produce large amounts of Th2 cytokines, which promotes B cell maturation and isotype switching. Spleens of these mice are enlarged enormously and lymphocytes infiltrate into different organs. It is not clear what causes the severe autoimmune phenotype in these mice. Defects in TCR signaling in LATY136F T cells might affect negative selection as demonstrated recently (31), leading to production of autoreactive T cells in these mice. In addition, the signaling defects might also affect development or survival of CD4^{+}CD25^{+} T reg cells that are capable of controlling the autoimmunity. In this study, we demonstrated that LATY136F mice lacked CD4^{+}CD25^{+} T cells expressing Foxp3 in peripheral lymphoid organs. Moreover, transfer of normal CD4^{+}CD25^{+} T reg cells in LATY136F mice prevented the lymphoproliferative syndrome. Ectopic expression of Foxp3 in LATY136F T cells conferred suppressive function to abrogate lymphoproliferative disease when transferred into LATY136F mice. Our findings provide the evidence that the proximal signaling pathways downstream of the TCR, specifically the LAT–PLC-γ1 interaction, control T cell homeostasis by regulating Foxp3 expression and development of T reg cells.

RESULTS

The absence of CD4^{+}CD25^{+} T cells in LAT^{Y136F} mice

The interaction of the TCR and self-peptides presented by MHC class II plays a critical role in development of CD4^{+}CD25^{+} T reg cells in the thymus and function in the periphery (6, 32), suggesting that TCR-mediated signaling might also be important in the development of T reg cells. We asked whether the severe autoimmune phenotype of the LAT^{Y136F} mice might be caused by a defect in development or function of T reg cells. We first examined whether CD4^{+}CD25^{+} T reg cells were present in LAT^{Y136F} mice. Cells from thymuses and spleens of LAT^{Y136F} and WT mice were analyzed for expression of CD4, CD8, and CD25 by FACS. As previously reported (29, 30), LAT^{Y136F} mice had a partial block in thymocyte development with accumulation of CD4^{-}CD8^{-} cells (Fig. 1 A). Splenomegaly and enlarged lymph nodes became obvious when they were 4–6 wk old. In the periphery, the CD4^{+} to CD8^{+} ratio was skewed toward CD4^{+} cells. Although discreet populations of CD4^{+}CD25^{+} and CD4^{+}CD25^{-} SP thymocytes could be observed in WT mice, most of CD4^{+} SP thymocytes in LAT^{Y136F} mice expressed low levels of CD25 (Fig. 1 A). The

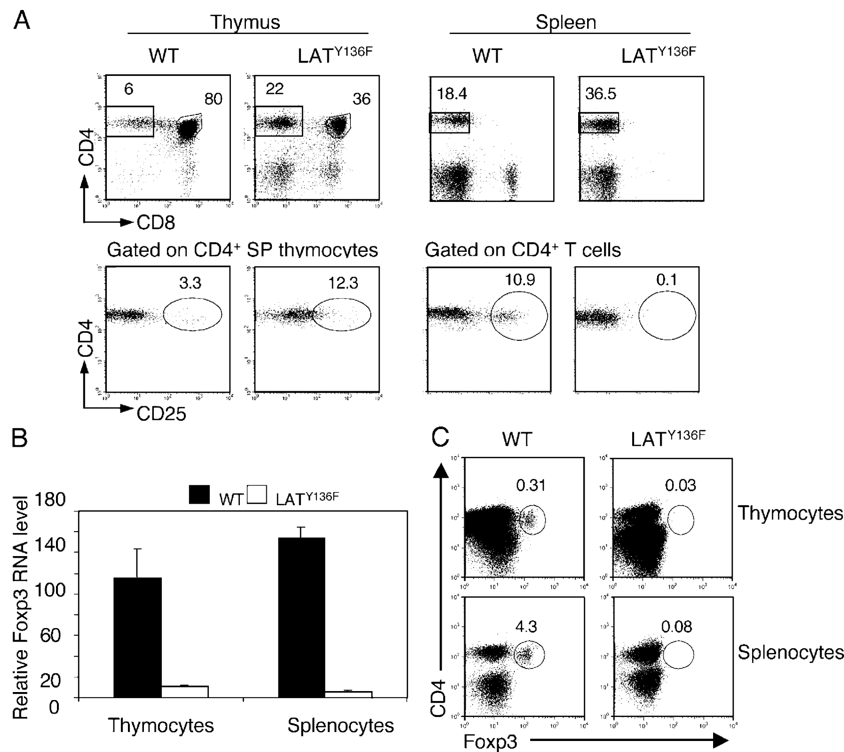

Figure 1. Disruption of the LAT–PLC-γ1 interaction leads to the absence of CD4^{+}CD25^{+} regulatory T cells in the periphery. (A) FACS analysis of CD4, CD8, and CD25 expression on thymocytes and splenocytes from 6-wk-old WT and LAT^{Y136F} mice. (B) Quantitative real-time PCR analysis of *Foxp3* expression in thymocytes and splenocytes. Total RNAs were prepared from 2×10^5 CD4^{+} SP thymocytes and CD4^{+} splenocytes sorted by FACS. The levels of *Foxp3* RNA were normalized with β-actin RNA. The data are representative of three independent experiments. (C) Expression of Foxp3 on CD4^{+} thymocytes and splenocytes from 6-wk-old WT and LAT^{Y136F} mice by intracellular staining with an anti-Foxp3 antibody.

aberrant expression of CD25 in LAT^{Y136F} thymocytes is likely caused by the partial block of thymocyte development in the DN3 (double negative) stage (29, 30), in which thymocytes are $CD25^{+}CD44^{-}$. Interestingly, double positive thymocytes in LAT^{Y136F} mice also expressed CD25 (not depicted). Thymocyte development in LAT^{Y136F} mice might proceed without down-regulation of CD25 expression.

Because thymocytes from LAT^{Y136F} mice expressed CD25, we next examined whether $CD4^{+}CD25^{+}$ T cells are present in the periphery. $CD4^{+}CD25^{+}$ T cells were clearly missing in the periphery of these mice (Fig. 1 A), although $CD4^{+}$ T cells from LAT^{Y136F} mice expressed high levels of GITR (not depicted). We further determined expression of Foxp3, an important transcription factor that is specifically expressed in $CD4^{+}CD25^{+}$ T reg cells, in LAT^{Y136F} T cells. $CD4^{+}$ SP thymocytes and $CD4^{+}$ splenocytes cells were sorted from both WT and LAT^{Y136F} mice. *Foxp3* expression was determined by RT-PCR. The level of *Foxp3* was decreased dramatically in $CD4^{+}$ SP LAT^{Y136F} thymocytes compared with that in $CD4^{+}$ SP WT thymocytes. Decrease in the level of *Foxp3* mRNA was also observed in $CD4^{+}$ T cells from the spleen of LAT^{Y136F} mice (not depicted). Further quantitation of *Foxp3* mRNA by real-time PCR showed that *Foxp3* mRNA level in $CD4^{+}$ SP thymocytes from LAT^{Y136F} mice was ~10-fold less than in WT thymocytes, and *Foxp3* RNA level in $CD4^{+}$ splenic T cells from LAT^{Y136F} mice was ~30-fold less (Fig. 1 B). To exclude the possibility that decreased Foxp3 expression in $CD4^{+}$ T cells from the mutant mice reflected dilution of $Foxp3^{+}$ cells because of the expansion of $CD4^{+}$ T cells, Foxp3 expression in thymocytes and splenocytes from WT and LAT^{Y136F} mice was examined by intracellular staining with an anti-Foxp3 antibody followed by flow cytometry. As shown in Fig. 1 C, only $CD4^{+}$ SP thymocytes and $CD4^{+}$ splenocytes derived from WT, not those from LAT^{Y136F} mice, expressed Foxp3. Thus, LAT^{Y136F} mice lack $CD4^{+}CD25^{+}$ T reg cells.

To exclude the possibility that the severe autoimmune conditions cause disappearance of T reg cells, we also examined development of T reg cells in younger mutant mice in which the autoimmune disease was not severe. As a result of a partial block in thymocyte development, very few $CD4^{+}$ or $CD8^{+}$ T cells were found in the peripheral lymphoid organs of 17-d-old mice (not depicted); however, they appeared in the spleens from 24-d-old mice (Fig. 2 A). Interestingly, a high percentage of $CD4^{+}$ SP thymocytes expressed CD25 (23.6%). There was also a higher percentage (1.23%; Fig. 2 A) of $CD4^{+}CD25^{+}$ splenocytes compared with 6-wk-old mice (0.1%; Fig. 1 A). However, these $CD4^{+}$ cells from the mutant mice did not express Foxp3 as indicated by intracellular staining (Fig. 2A). We also performed a mixed bone marrow chimeras experiment. Lethally irradiated $LAT^{-/-}$ mice were transferred with mixed bone marrow cells from WT $Thy1.1^{+}$ and LAT^{Y136F} $Thy1.2^{+}$ mice. 6 wk after transfer, these mice showed no apparent signs of lymphoproliferative disease, whereas $LAT^{-/-}$ mice received bone marrow cells from LAT^{Y136F} mice developed the autoimmune disease

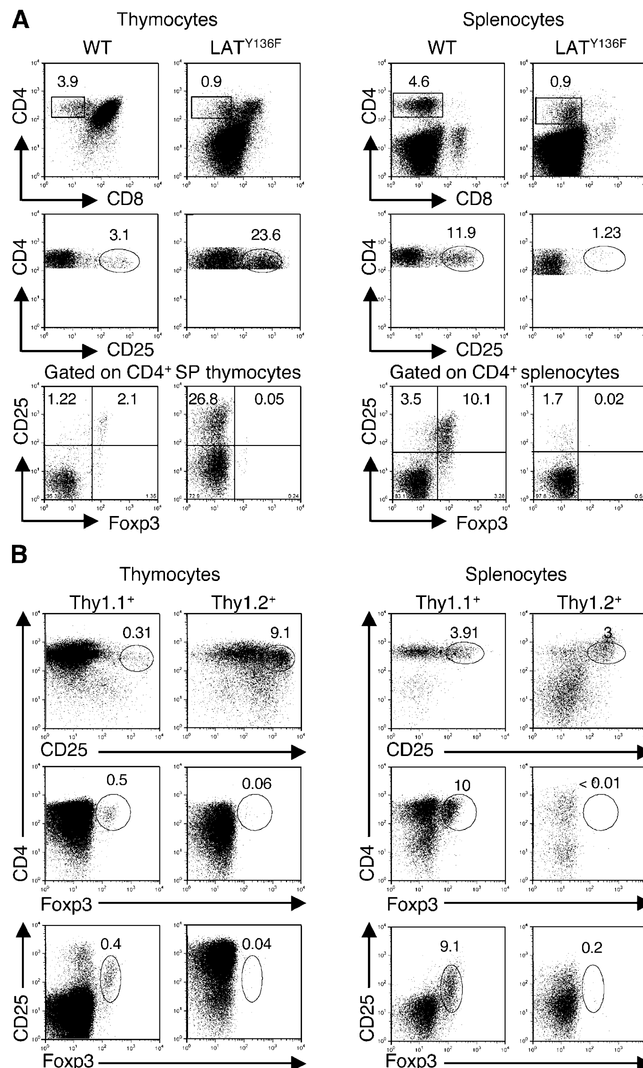

Figure 2. CD25 and Foxp3 expression in LAT^{Y136F} T cells in young mice and mice with mixed bone marrow chimeras. (A) FACS analysis of CD4, CD8, CD25, and Foxp3 in thymocytes and splenocytes from 24-d-old WT and LAT^{Y136F} mice. (B) Irradiated $LAT^{-/-}$ mice were reconstituted with mixed bone marrow cells from $Thy1.2^{+}$ LAT^{Y136F} mice (3.0×10^{6} cells) and congenic B6 $Thy1.1^{+}$ mice (1.5×10^{6} cells) after T cell depletion. 6 wk after reconstitution, thymocytes and splenocytes from these mice were analyzed. Cells were stained with APC anti-CD4, Texas red anti-CD25, PE-Cy5 anti-CD8, PE anti-Thy1.2 PE, biotin anti-Thy1.1, and strepavidin PE-Cy7 and FITC anti-Foxp3. FACS plot shown is a representative of analysis of six mice.

like LAT^{Y136F} mice (not depicted). Because of the partial block in thymocyte development in LAT^{Y136F} mice, fewer $Thy1.2^{+}$ T cells than $Thy1.1^{+}$ T cells were detected even though more bone marrow cells from LAT^{Y136F} $Thy1.2^{+}$ mice were transferred. As expected, WT $Thy1.1^{+}$ bone marrow cells gave normal population of $CD4^{+}CD25^{+}$ T cells. A small population of $Thy1.1^{+}$ T cells expressed Foxp3, and these $Foxp3^{+}$ cells also expressed CD25 (Fig. 2 B). In contrast, very few $Thy1.2^{+}$ cells in thymuses and spleens, if any,

expressed Foxp3. Interestingly, different from the 6-wk-old LAT^{Y136F} mice with the autoimmune disease, a large percentage of Thy1.2$^+$ CD4$^+$ splenocytes expressed CD25. It is possible that CD25 expression is down-regulated with progression of the disease. Because these Thy1.2$^+$$LAT^{Y136F}$ T cells did not express Foxp3, they likely represented activated T cells. Collectively, these results indicate that LAT^{Y136F} mice, which express a LAT mutant that does not bind PLC-γ1, have a defect in Foxp3 expression and development of CD4$^+$CD25$^+$ T reg cells.

Adoptive transfer of CD4$^+$CD25$^+$ T reg cells

To investigate whether the absence of CD4$^+$CD25$^+$ T reg cells is indeed one of the underlying mechanisms responsible for the lymphoproliferative syndrome in LAT^{Y136F} mice, we purified CD4$^+$CD25$^+$ or CD4$^+$CD25$^-$ T cells from congenic Thy1.1$^+$ mice by FACS sorting. 2–3 × 10^5 CD4$^+$CD25$^+$ or CD4$^+$CD25$^-$ Thy1.1$^+$ T cells were transferred into 3-d-old Thy1.2$^+$ LAT^{Y136F} neonatal mice by i.p. injection. These mice were analyzed at 7 wk after injection. Untreated LAT^{Y136F} mice clearly developed a pathological lymphoproliferative syndrome at 7 wk of age as shown before (29, 30) (Fig. 3 A). Compared with untreated LAT^{Y136F}mice, LAT^{Y136F} mice that received Thy1.1$^+$CD4$^+$CD25$^+$ T cells had a normal size spleen and lymph nodes. In contrast, LAT^{Y136F} mice injected with CD4$^+$CD25$^-$ T cells developed a lymphoproliferative syndrome similar to untreated LAT^{Y136F} mice (Fig. 3 A).

We further examined donor cell engraftment and expansion of adoptively transferred cells in LAT^{Y136F} recipient mice. In normal mice, CD4$^+$CD25$^+$ T cells comprise ~5–10% of the peripheral CD4$^+$ T cells or ~1–2% of total cells in lymph node and spleen. FACS analysis 7–9 wk after adoptive transfer revealed that 1–2% of the total cells in the lymph node and spleen of LAT^{Y136F} mice adoptively transferred with CD4$^+$CD25$^+$ T cells were of donor origin, whereas very few donor cells were detected in the thymus (not depicted). This level of engraftment corresponded to ~4–9-fold expansion of the initial donor cell inoculum of 2 × 10^5 cells. A similar degree of donor CD4$^+$CD25$^+$ T cell expansion was observed after injection of CD4$^+$CD25$^+$ T cells into *$Foxp3^{sf}$* neonates (21). Likewise, CD4$^+$CD25$^-$ injected into LAT^{Y136F} neonates also underwent a considerable degree of expansion as the number of Thy1.1$^+$CD4$^+$CD25$^-$ T cells increased

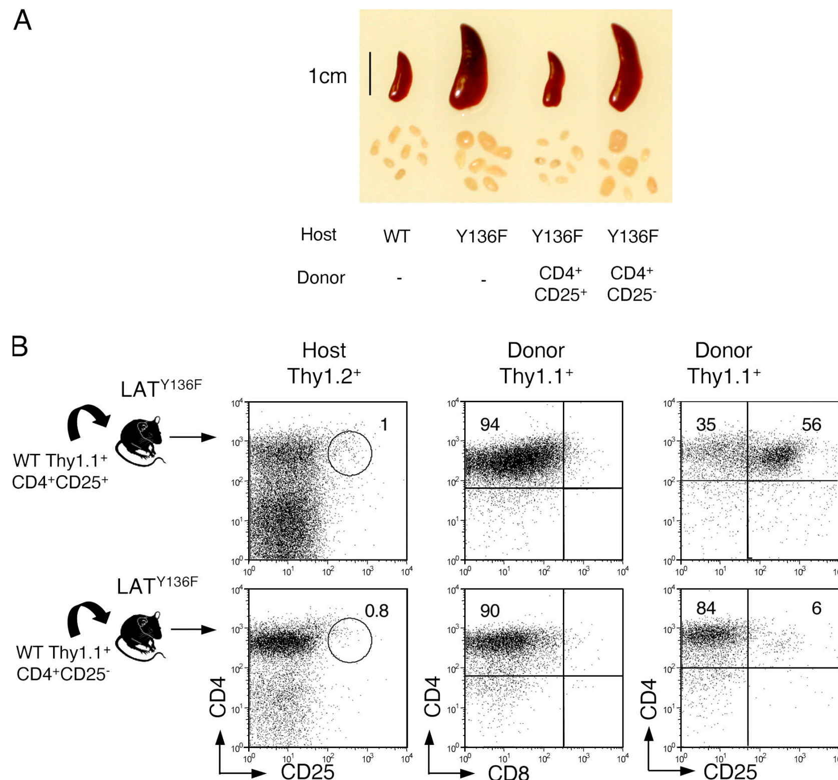

Figure 3. Neonatal adoptive transfer of normal CD4$^+$CD25$^+$ regulatory T cells prevents the lymphoproliferative syndrome in LAT^{Y136F} mice. Neonatal Thy1.2$^+$ LAT^{Y136F} mice were adoptively transferred with 2–3 × 10^5 CD4$^+$CD25$^+$ or CD4$^+$CD25$^-$Thy1.1$^+$ T cells and analyzed 7 wk later. (A) Photographs of spleens and lymph nodes from 7-wk-old WT, LAT^{Y136F}, LAT^{Y136F} received CD4$^+$CD25$^+$, and LAT^{Y136F} received CD4$^+$CD25$^-$ T cells. This picture is a representative of six experiments. The numbers of splenocytes in these mice were 9.3 × 10^7, 34.9 × 10^7, 9.8 × 10^7, and 31.0 × 10^7, respectively. (B) FACS analysis of donor cell engraftment. splenocytes were stained with FITC anti-Thy1.1 or FITC anti-Thy1.2, PE anti-CD25, and APC anti-CD4. The percentage of cells in the gated region is shown in the dot plot. FACS plot shown is a representative of five independent experiments.

at least 20-fold (not depicted). In contrast, only very few donor-derived $CD4^+CD25^+$ and $CD4^+CD25^-$ cells were detectable when they were transferred into age-matched WT recipients, which had normal T cell compartment. This observation reconciles with the possibility proposed by Fontenot et al. that donor $CD4^+CD25^+$ T cells can expand to fill the available homeostatic niche despite a skewed T cell compartment in these mice (21). In addition, adoptive transfer of WT $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells into 3-wk-old LAT^{Y136F} mice that have not fully developed lymphoproliferative disease also resulted in expansion of both populations of donor-derived T cells (not depicted). However, only the transfer of $CD4^+CD25^+$ T cells was able to partially suppress lymphoproliferative disorder in 3-wk-old LAT^{Y136F} recipients (not depicted). Neonatal adoptive transfer might provide donor-derived $CD4^+CD25^+$ T cell population with sufficient time to proliferate and suppress LAT^{Y136F} T cells to fully suppress lymphoproliferative disorder. FACS analysis showed that in both groups of treated LAT^{Y136F} mice >99% of the engrafted donor cells were still $CD4^+$ T lymphocytes as expected. At least 75% of the donor $CD4^+$ T cells expressed high levels of CD25 in the LAT^{Y136F} mice transferred with $CD4^+CD25^+$ T cells, whereas the absolute majority of the donor $CD4^+$ T cells were still $CD25^-$ in mice that received $CD4^+CD25^-$ T cells. Adoptive transfer of either population of donor T cells had no effect on expression of CD25 on the host T cells (Fig. 3 B).

No effect of donor T cells on the phenotypes of recipient T cells

To evaluate whether adoptive transfer of normal $CD4^+CD25^+$ T cells could change activated phenotypes or other intrinsic properties in the recipient T cells leading to suppression of the lymphoproliferative disorder, thymocytes and splenocytes from LAT^{Y136F} mice that received the adoptive transfer were analyzed and compared with those of untreated WT and LAT^{Y136F} mice. Thymocyte development in LAT^{Y136F} mice that received $CD4^+CD25^+$ T cells was similar to that in untreated LAT^{Y136F} mice (Fig. 4 A). In fact, no substantial number of donor T cells was detected in the thymus of these mice (not depicted). However, the LAT^{Y136F} mice transferred with $CD4^+CD25^+$ T cells had a significantly lower percentage of $CD4^+$ T cells in the spleen (∼3%) compared with untreated WT (27%) and LAT^{Y136F} (56%) mice. Despite a substantial reduction in the number of $CD4^+$ T cells in LAT^{Y136F} mice, donor $CD4^+CD25^+$ T cells did not alter the activated phenotypes of LAT^{Y136F} T cells as T cells from both treated and untreated LAT^{Y136F} mice were identical ($CD25^-$ $TCR\beta^{low}CD62L^{low}CD44^{high}$; Fig. 4 B). In addition, T cells from treated LAT^{Y136F} mice failed to mobilize Ca^{2+}-like T cells from untreated LAT^{Y136F} mice (not depicted). These results indicate that donor $CD4^+CD25^+$ T cells did not change the activated phenotypes of LAT^{Y136F} T cells based on these parameters we tested. Instead, $CD4^+CD25^+$ T reg cells transferred into LAT^{Y136F} mice likely suppress expansion of $CD4^+$ LAT^{Y136F} T cells.

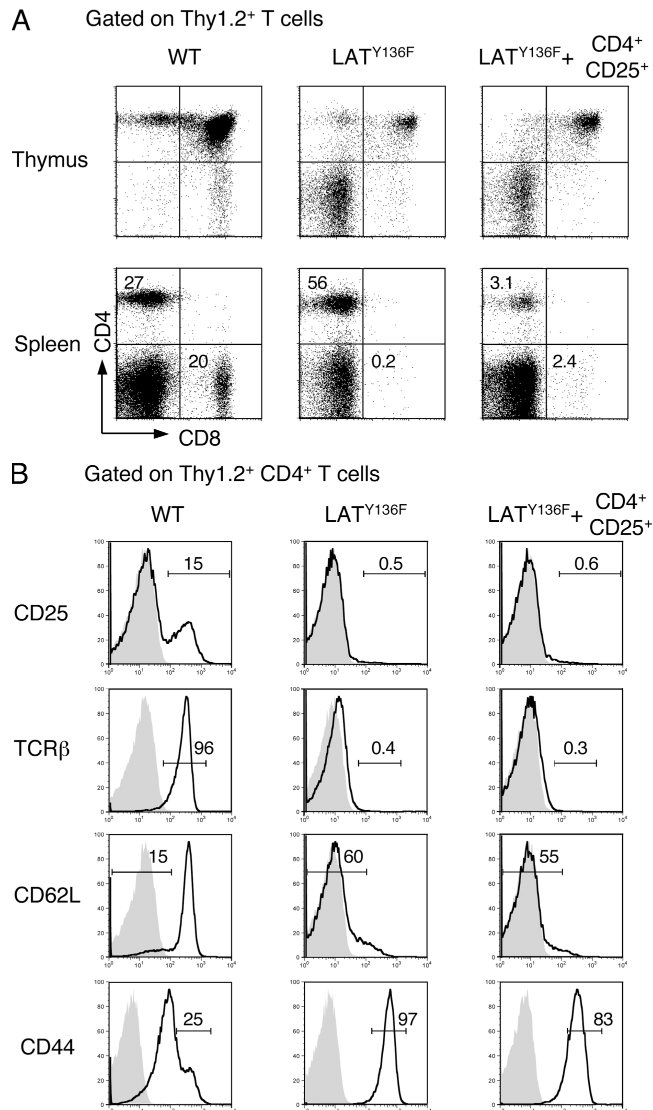

Figure 4. Analysis of host-derived cells in LAT^{Y136F} mice after adoptive transfer of $CD4^+CD25^+$ T cells. (A) FACS analysis of thymocytes and splenocytes from untreated and treated LAT^{Y136F} mice 7 wk after injection. (B) Expression of CD25, TCR-β, CD62L, and CD44 in host-derived $Thy1.2^+$ $CD4^+$ T cells from control and treated LAT^{Y136F} mice. Numbers in the dot plots or histograms indicate the percentage of cells within the designated gate. The shaded area in the histogram represents FACS staining of samples with isotype control antibodies. FACS plot shown is a representative of five independent experiments.

Rescue of the autoimmune phenotype in LAT^{Y136F} mice

To determine if transfer of $CD4^+CD25^+$ T cells into LAT^{Y136F} mice could correct the autoimmune disease, we performed a histological analysis of spleen, liver, and kidney from those mice. Histological sections from untreated LAT^{Y136F} mice showed disorganized B and T cell zones in the spleen, lymphocyte infiltration in the majority of portal veins and liver sinusoids, and immune complex deposition in the glomeruli of the kidneys (Fig. 5 A). In contrast, tissue sections from LAT^{Y136F} mice treated with $CD4^+CD25^+$

T cells appeared relatively normal, although many Thy1.2$^+$ T cells did appear in the B cell zone of the spleen (Fig. 5 A). Histological analysis of the spleen, liver, and kidney of LAT^{Y136F} mice that received CD4$^+$CD25$^-$ T cells exhibited lymphoproliferative diseases similar to untreated LAT^{Y136F} mice (not depicted).

Examination of serum antinuclear (not depicted) and anti–double stranded DNA antibodies revealed that LAT^{Y136F} mice treated with CD4$^+$CD25$^+$ T reg cells had comparable levels to WT controls (Fig. 5 B). These data were in agreement with a reduction in the number of hyperactivated B cells (MHC class II^{hi} or IgM$^-$B220$^+$) in LAT^{Y136F} mice treated with CD4$^+$CD25$^+$ T cells (Fig. 5 C). In addition, the concentration of IgG1 (Fig. 5 D) or IgE (not depicted), two antibody isotypes dramatically elevated in LAT^{Y136F} mice as a result of increased B cell maturation (29), was significantly reduced in mice treated with CD4$^+$CD25$^+$ T cells. Collectively, our data indicate that adoptive transfer of CD4$^+$CD25$^+$ T cells into LAT^{Y136F} neonates can protect LAT^{Y136F} mice from further developing lymphoproliferative syndrome.

Up-regulation of granzymes in transferred CD4$^+$CD25$^+$ T cells

CD4$^+$CD25$^+$ T reg cells are capable of suppressing autoimmune disease; however, how these cells exert their suppressive function is still not clear. Inhibitory cytokines such as IL-10 and TGF-β are considered to be the key molecules involved in T reg cell–mediated immunosuppression (33). Recently, it was reported that CD4$^+$CD25$^+$ T cells up-regulate granzyme B in vitro upon stimulation with anti-CD3 and IL-2 (34). Whether this happens in vivo has not been demonstrated. We asked whether CD4$^+$CD25$^+$ T reg cells up-regulate granzymes (A and B) or inhibitory cytokines when they are transferred into LAT^{Y136F} mice. At 7 wk after adoptive transfer of CD4$^+$CD25$^+$ or CD4$^+$CD25$^-$ Thy1.1$^+$ T cells into LAT^{Y136F} mice, donor cells were reisolated from these mice by FACS sorting. Because these Thy1.1$^+$CD4$^+$CD25$^+$ T cells were placed in the autoimmune environment and capable of correcting the disease, they must be activated to exert their suppressive function. They were labeled as "activated" in Fig. 5 E. We also isolated Thy1.2$^+$CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells from WT mice as "resting" T reg

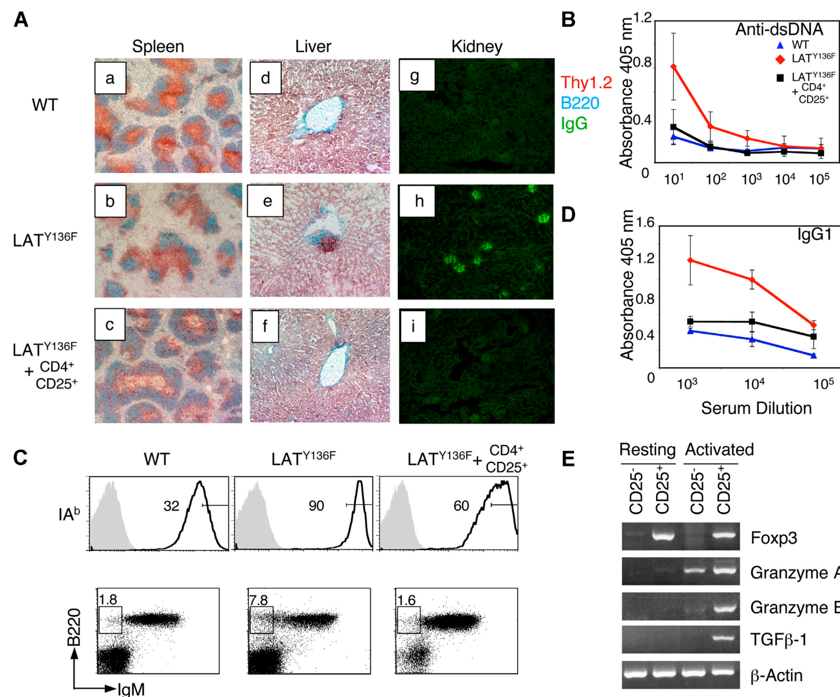

Figure 5. Effect of adoptive transfer of normal CD4$^+$CD25$^+$ T cells into LAT^{Y136F} mice. (A) Immunohistochemistry staining of frozen sections of spleen (a–c), liver (d–f), and fluorescence staining of kidney (g–i). Frozen sections were prepared from the spleens, livers, and kidneys of 7- to 9-wk-old untreated controls and treated LAT^{Y136F} mice. Spleen and liver sections were stained with anti-B220 (blue) and anti–TCR-β (red). Kidney sections were stained with anti–mouse IgG-FITC (green) for glomeruli. Spleen and kidney photographs were taken at ×10 magnification. Liver photographs were taken at ×100 magnification. (B) Anti–double-stranded DNA antibody titers in control untreated and LAT^{Y136F} mice treated with CD4$^+$CD25$^+$ regulatory T cells. (C) Decrease in hyperactivated B lymphocyte numbers (B220$^+$MHCII^{hi} and B220$^+$IgM^{lo}) in treated LAT^{Y136F} mice. (D) Reduced IgG1 production. IgG1 from serum samples of 7-wk-old WT, LAT^{Y136F}, and treated LAT^{Y136F} mice were subjected to serial dilution and quantitated by ELISA. (E) Expression of Foxp3, granzyme A, granzyme B, and TGF-β. Thy1.2$^+$ T cells (CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$) were freshly isolated from normal C57BL/6 mice by FACS sorting. Thy1.1$^+$ T cells were sorted from LAT^{Y136F} mice received adoptive transfer of CD4$^+$CD25$^+$ or CD4$^+$CD25$^-$ T cells from Thy1.1$^+$ mice, respectively. Amounts of cDNAs used RT-PCR were normalized by the level of β-actin. Data in B and D represent mean ± SD of serum samples from five groups of mice.

cells and Thy1.2$^+$CD4$^+$CD25$^-$ T cells as a negative control. Total RNAs were prepared from these cells and used in RT-PCR.

Donor-derived Thy1.1$^+$CD4$^+$CD25$^+$ T cells expressed a high level of Foxp3 similar to Thy1.2$^+$CD4$^+$CD25$^+$ cells directly isolated from WT mice. Thy1.1$^+$ or Thy1.2$^+$ CD4$^+$CD25$^-$ T cells did not have a substantial level of Foxp3 expression (Fig. 5 E). Interestingly, Thy1.1$^+$CD4$^+$CD25$^+$ T cells from neonatally injected LAT^{Y136F} mice expressed high levels of granzyme A, granzyme B, and TGF-β RNAs compared with Thy1.2$^+$CD4$^+$CD25$^+$ T cells (Fig. 5 E), whereas their IL-10 expression was comparable to that of resting T reg cells (not depicted). This result is consistent with recent findings in which a granzyme B–dependent mechanism was identified as contact-mediated suppression by CD4$^+$CD25$^+$ T cells in vitro (34). Increased TGF-β expression could also function to suppress the expansion of CD4$^+$ T cells from LAT^{Y136F} mice. It is possible that transferred Thy1.1$^+$CD4$^+$CD25$^+$ T cells use both cytokine-mediated and contact-mediated mechanisms to suppress hyperproliferative host CD4$^+$ T cells in vivo. Although we have not demonstrated that increased granzymes or TGF-β indeed mediates suppression in LAT^{Y136F} mice, our results show that T reg cells indeed up-regulate these proteins in the autoimmune environment.

Ectopic expression of Foxp3 in LAT^{Y136F} T cells

Ectopic expression of Foxp3 was previously shown to be sufficient to activate a program of immunosuppression in CD4$^+$CD25$^-$ T cells (21). Because Foxp3 expression was significantly decreased in LAT^{Y136F} T cells (Fig. 1, B and C), we asked whether reexpression of Foxp3 in LAT^{Y136F} T cells could confer the regulatory function that normal CD4$^+$CD25$^+$ T cells have. Because LAT^{Y136F} T cells express low levels of TCRs on their surface, they were difficult to be activated via the TCR. Instead, LAT^{Y136F} T cells were stimulated with PMA and ionomycin and cultured in the presence of IL-2 for 48 h before transduction with retroviruses expressing Foxp3 and green fluorescence protein (GFP) (pHSpG-Foxp3) or GFP alone (pHSpG-Empty) (Fig. 6 A). After culture for an additional 48 h postretroviral transduction, GFP$^+$CD4$^+$ T cells were FACS sorted, and $2-3 \times 10^5$ purified GFP$^+$CD4$^+$ LAT^{Y136F} T cells were i.p. injected into 3-d-old LAT^{Y136F} neonates. Expression levels of TCR-β and the GITR on transduced LAT^{Y136F} CD4$^+$ T cells with or without Foxp3 were similar, whereas those of CD25 were slightly higher on pHSpG-Foxp3–transduced cells (Fig. 6 A). Expression of high levels of CD25 in these cells is likely a consequence of stimulation by PMA and ionomycin. 6 wk posttransfer, spleens and lymph nodes were harvested and examined. Mice receiving purified LAT^{Y136F} T cells expressing only GFP developed a severe lymphoproliferative syndrome like untreated LAT^{Y136F} mice (Fig. 6 B). In contrast, mice that received LAT^{Y136F} T cells expressing Foxp3 and GFP exhibited no sign of lymphoproliferative disease as judged by the gross appearance of secondary lymphoid organs (Fig. 6 B). Analysis of GFP$^-$ T cells from mice received CD4$^+$ LAT^{Y136F} T cells expressing Foxp3 and GFP revealed a dramatic decrease in CD4$^+$ T cells similar to that observed in LAT^{Y136F} mice that received normal CD4$^+$CD25$^+$ T cells. On the other hand, GFP$^-$ host T cells from LAT^{Y136F} mice treated with CD4$^+$ LAT^{Y136F} T cells expressing GFP alone were predominantly CD4$^+$ similar to those in LAT^{Y136F} mice (Fig. 6 C). These data indicate that reconstitution of Foxp3 expression in LAT^{Y136F} T cells is sufficient to induce suppressive function and protect LAT^{Y136F} mice from lymphoproliferative disease.

DISCUSSION

In this study, we demonstrated an essential role of the adaptor protein LAT in Foxp3 expression and CD4$^+$CD25$^+$ T reg cell development. In LAT^{Y136F} mice with a severe lymphoproliferative disease, CD4$^+$CD25$^+$ cells were nearly absent in peripheral lymphoid organs. Foxp3 expression was also dramatically decreased in the LAT^{Y136F} T cells. Interestingly, in young LAT^{Y136F} mice CD4$^+$CD25$^+$ cells could be found; however, they did not express Foxp3. Similar results were also seen in LAT$^{-/-}$ mice that received mixed WT and LAT^{Y136F} bone marrow chimeras. These data indicate that the LAT–PLC-γ1 interaction is required for Foxp3 expression and T reg cell development. Severe lymphoproliferative disease in LAT^{Y136F} knock-in mice could be prevented by transfer of normal CD4$^+$CD25$^+$ T reg cells but not CD4$^+$CD25$^-$ T cells. Our results indicate that the lymphoproliferative disease associated with the LAT^{Y136F} mutation is not only caused by abrogation of central tolerance (31), but also by a breakdown in peripheral tolerance caused by a severe block of CD4$^+$CD25$^+$ T reg cell development.

Analysis of donor cell engraftment indicates that CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells are able to proliferate and expand in LAT^{Y136F} mice. Two studies using IL-2Rβ– and Foxp3-deficient mice have previously demonstrated a similar expansion after the transfer of CD4$^+$CD25$^+$ T reg cells (12, 21). A rich Th2 cytokine environment, a consequence of hyperactivated LAT^{Y136F} CD4$^+$ T cells, may provide an ideal environment to support the expansion of transferred CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells. In LAT^{Y136F} mice, at 2–3 wk after birth both CD4$^+$ and CD8$^+$ T cells begin to fill in peripheral lymphoid organs as a result of a partial block in thymocyte development. Nonselective expansion of transferred T cells might be caused by the lymphopenic environment in neonatal LAT^{Y136F} mice. Adoptive transfer of CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells into 3-wk-old LAT^{Y136F} recipient mice also allowed engraftment and expansion of both T cell populations. Thus, it is also possible that this expansion of transferred T reg cells may result from a proliferative response to fill a homeostatic niche for CD4$^+$CD25$^+$ T cells (10, 35). Although donor-derived CD4$^+$CD25$^-$ T cells expanded upon transfer into LAT^{Y136F} neonates, only transfer of CD4$^+$CD25$^+$ T cells into LAT^{Y136F} mice rescued the lymphoproliferative disease. Even though CD4$^+$CD25$^-$ T cells could convert into CD4$^+$CD25$^+$ T cells upon homeostatic proliferation (36), CD4$^+$CD25$^-$

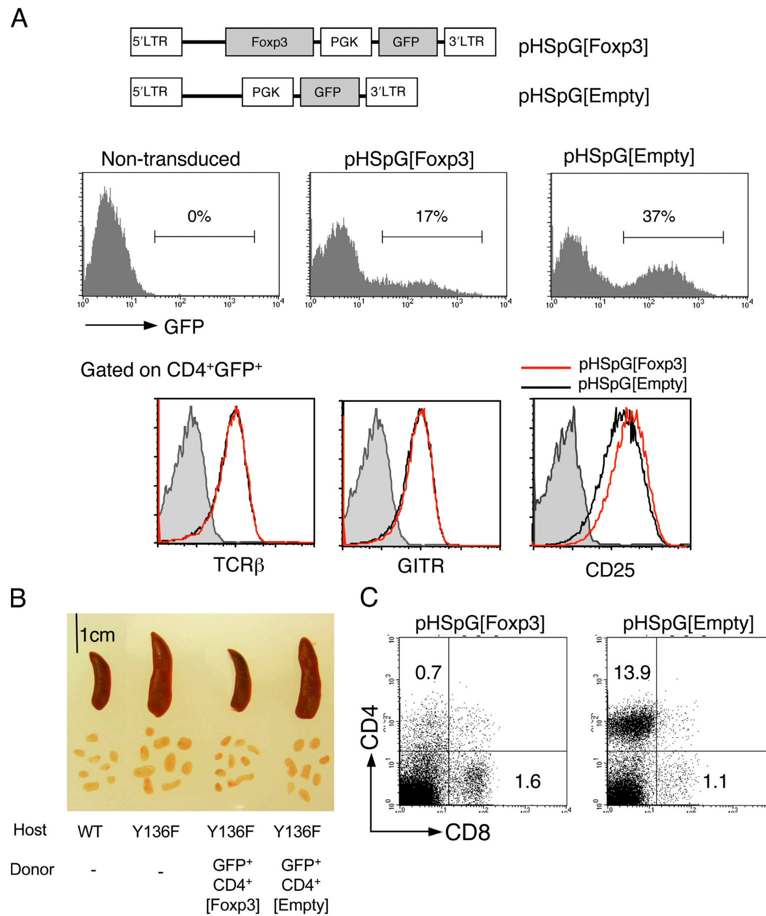

Figure 6. Expression of Foxp3 confers suppressive function in LAT^{Y136F} CD4$^+$CD25$^-$ T cells. (A) Retroviral constructs and retroviral transduction efficiency of CD4$^+$LAT^{Y136F} cells before neonatal injection. Retrovirally transduced cells were stained with fluorescent-conjugated APC anti-CD4 and PE anti–TCR-β, PE anti-GITR, and PE anti-CD25 antibodies, respectively. Gated GFP$^+$CD4$^+$ cells were analyzed for expression of TCR-β, GITR, and CD25. (B) Representative spleens and lymph nodes of 6-wk-old untreated WT, LAT^{Y136F} mice and treated LAT^{Y136F} mice injected with either 2 × 10^5 T cells expressing GFP alone or GFP and Foxp3. The picture shown is a representative of four experiments. (C) FACS analysis of CD4 and CD8 expression on splenocytes of recipient origin.

T cells injected into neonatal or 3-wk-old LAT^{Y136F} mice neither converted into a considerable number of CD4$^+$CD25$^+$ T cells nor rescued the lymphoproliferative disease.

The appearance of CD4$^+$CD25$^+$ LAT^{Y136F} T cells in peripheral lymphoid organs of mixed bone marrow chimeras or young mutant mice was unexpected because CD25 expression was almost absent on CD4$^+$ T cells from LAT^{Y136F} mice older than 6 wk. However, in mice that received treatment of CD4$^+$CD25$^+$ at the neonatal stage CD25 expression on LAT^{Y136F} CD4$^+$ T cells was still missing. Regardless of CD25 expression in LAT^{Y136F} T cells from different mice, Foxp3 expression was not detected in these cells, suggesting that these cells are likely activated T cells, not real T reg cells. It is possible that IL-2 produced by WT T cells might induce or maintain a high expression level of CD25 on LAT^{Y136F} T cells or the autoimmune environment in LAT^{Y136F} mice may cause T cells to gradually lose CD25 expression. Neonatally injected T reg cells, which do not secret IL-2 (4, 22, 37), failed to restore CD25 expression on LAT^{Y136F} T cells in these mice and provided only protective function against lymphoproliferative disease. Previous studies show that Foxp3 is up-regulated upon activation of CD4$^+$CD25$^+$ T reg cells, and that ectopic Foxp3 expression confers suppressor function upon peripheral CD4$^+$CD25$^-$ T cells (21). Likewise, ectopic expression of Foxp3 conferred LAT^{Y136F} CD4$^+$ T cells a regulatory function that prevents autoimmunity in these knock-in mice. Although ectopic expression of Foxp3 had no effect on the levels of both TCR-β and GITR, CD25 was slightly up-regulated on transduced CD4$^+$ T cells of LAT^{Y136F} mice. It is difficult to conclude the effect of Foxp3 on CD25 in our experiments because we had to activate LAT^{Y136F} T cells with PMA and ionomycin, which up-regulate CD25 in vitro, before transducing these cells with retroviruses. Nevertheless, our results suggest that Foxp3-mediated suppression does not require normal function of LAT, perhaps independent of the TCR. Our data strongly support the notion that Foxp3 is a master regulator gene that controls suppressor function in CD4$^+$CD25$^+$ T cells.

The LAT–PLC-γ1 interaction is important in TCR-mediated Ca^{2+} flux and MAPK activation (38, 39). LAT^{Y136F} T cells have abrogated Ca^{2+} flux although TCR-mediated MAPK activation is normal (30). As the influx of extracellular Ca^{2+} after TCR engagement has been implicated in influencing the outcome of both positive and negative selection (40–42), it is also possible that TCR-mediated Ca^{2+} mobilization and further NFAT activation might be required for induction of Foxp3 expression. Interestingly, the phenotype of LAT^{Y136F} mice resembles that of mice lacking NFATc2 and NFATc3 (43), which suggests that the autoimmune disease in these mice may be attributed, at least in part, to a decrease in NFAT activation. However, it has been demonstrated that combined NFATc2/c3 deficiency has no effect on development and function of $CD4^+CD25^+$ T cells but renders $CD4^+CD25^-$ T cells unresponsive to suppression (44). Based on these findings, we speculate that the LAT–PLC-γ1 interaction provides signals other than NFAT activation to induce Foxp3 expression. Because LAT^{Y136F} $CD4^+$ T cells can be suppressed in vivo, NFATc2/c3, which are required for $CD4^+CD25^-$ T cells to be suppressed, might be activated independent of the TCR. Although our results indicated that the LATY136F mutation affected Foxp3 expression and T reg cell development, we cannot rule out the possibility that the LAT^{Y136F} T reg cells can develop, but they might not be able to survive in these mice. In addition, because T cell development is partially blocked in the LAT^{Y136F} mice, it is possible that the defect in Foxp3 expression and T reg cell development might be indirect consequences of the block in thymocyte development. The signaling pathways that link the LAT–PLC-γ1 association and Foxp3 expression or T reg cell development remain to be explored in the future.

A dramatic decrease in peripheral $CD4^+$ T cells in treated LAT^{Y136F} mice strongly indicates that these autoreactive $CD4^+$ T cells are the targets of $CD4^+CD25^+$ T cell–mediated suppression. Our data show that donor-derived $CD4^+CD25^+$ T cells from treated LAT^{Y136F} mice expressed high levels of granzyme A, granzyme B, and TGF-β compared with $CD4^+CD25^+$ T cells isolated directly from WT mice (Fig. 5 E). In normal mice, maintenance of central tolerance mechanisms by negative selection leaves only a scanty number of these potentially harmful T cells in the periphery. The majority of T reg cells may never encounter these self-reactive T cells and thus remain at the resting status. Once placed or exposed to the autoimmune conditions, $CD4^+CD25^+$ T reg cells may trigger both contact-dependent and cytokine-mediated mechanisms by secretion of granzymes and TGF-β. Whether these proteins indeed function to kill or suppress $CD4^+$ LAT^{Y136F} T cells in vivo remains to be determined in the future. In conclusion, our study indicates that the proximal signaling pathways downstream of the TCR mediated by the LAT–PLC-γ1 interaction play an important role in $CD4^+CD25^+$ T cell development and opens up the question of what might be the missing link between TCR and Foxp3 induction.

MATERIALS AND METHODS

Mice. All mice were used in accordance with the 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. Mice were housed in specific pathogen-free conditions at the Duke University Animal Care facility.

Antibodies. Streptavidin-conjugated Texas red and PE-Cy7 and biotinylated FITC, PE, and PE-Cy5, APC-conjugated antibodies to TCR-β, CD4, CD8α, CD25, B220, CD62L, CD44, GITR, Thy1.1, Thy1.2, IgM, IA^b, and mouse Ig were purchased from BD Biosciences. The anti-Foxp3 antibody was from eBioscience.

Cell purification. Cells were maintained in complete RPMI 1640 medium with 10% FCS. To isolate $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells, splenocytes and lymph node cells were isolated. $CD8^+$ T and B cells were depleted by magnetic beads using biotin-conjugated anti-CD8 and anti-B220. The enriched $CD4^+$ lymphocytes were stained with FITC–anti-CD4, PE–anti-CD25, and 7AAD. $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were purified by cell sorting using a FACSVantage SE flow cytometer (BD Biosciences).

Neonatal transfer of $CD4^+$ T cells. $2–3 \times 10^5$ WT $Thy1.1^+$-marked $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells were injected i.p. into 3-d-old ($Thy1.2^+$) LAT^{Y136F} pups or 3-wk-old LAT^{Y136F} mice. These LAT^{Y136F} pups were derived from breeding $LAT^{+/Y136F}$ females with $LAT^{-/-}$ males. Injected pups were analyzed at 7−9 wk after the adoptive transfer. Donor cell recovery was calculated based on the total number of lymphocytes multiplied by the percentage of $Thy1.1^+$ cells as determined by FACS analysis.

Mixed bone marrow transfer. T cell–depleted bone marrow cells from $Thy1.2^+$ LAT^{Y136F} mice (3.0×10^6 cells) were mixed with $Thy1.1^+$ congenic mice (1.5×10^6 cells) and were then injected i.v. into irradiated $LAT^{-/-}$ mice (900 rads). 6 wk after bone marrow reconstitution, thymuses and spleens were harvested and analyzed by FACS.

Retroviral transduction of LAT^{Y136F} $CD4^+CD25^-$ T cells. pHSpG and pHSpG/Foxp3 retroviral vectors were used to transfect the Phoenix-ecotropic virus packaging cell line using the calcium phosphate method to produce recombinant retroviruses. To transduce T cells from LAT^{Y136F} mice, splenocytes from LAT^{Y136F} mice were first activated using 40 ng ml^{-1} PMA and 0.5 μg ml^{-1} ionomycin and recombinant mouse IL-2 (100 ng ml^{-1}) for 36 h. Activated lymphocytes were then transduced by mixing with the retroviral supernatant in the presence of 8 μg ml^{-1} polybrene and recombinant mouse IL-2 (100 ng ml^{-1}). Cells were then centrifuged at 1,300 g for 2 h at 22°C. After culturing those cells at 37°C for 24 h, the transduction procedure was repeated. At 48 h after viral transduction, GFP^+CD4^+ T cells were sorted by FACS and $2-3 \times 10^5$ GFP^+CD4^+ T cells were injected i.p. into LAT^{Y136F} neonates. At 6 wk after injection, lymph node and spleen cells were isolated and analyzed.

Real-time quantitative PCR and RT-PCR. Total RNAs were extracted with the Trizol reagent (Invitrogen) and reverse transcribed using Superscript II reverse transcriptase (Invitrogen). cDNAs were then used as templates in PCR amplification with Taq polymerase. The *Foxp3* mRNA level was quantified using the LightCycler system (Roche). The primer pairs used in real-time PCR were the following: β-actin, 5′-ACTCCTATGTG-GGTGACGAG-3′, 5′-CAGGTC-CAGACGCAGGATGGC-3′; Foxp3, 5′-CCCAGGAAAGACAGCAACCTT-3′,5′-TTCTCA-CAACCAGGC-CACTTG-3′. The primer pairs used in RT-PCR were the following: Foxp3, 5′-CAGCTGCCTACAGTGCCCCTAG-3′, 5′-CATTTGCC-AGCAGTGGGTAG-3′; granzyme A, 5′-CTCAAGACCGTATATGG-CTCT-3′, 5′-CCTGCACAAATCATGTTTAGT-3′; granzyme B, 5′-ACTTTCGATCAAGGATCAGCA-3′, 5′-ACTGTCAGCTCAAC-CTCTTGT-3′; TGF-β1, 5′-TGCTGCTTTCTCCCTCAACCT-3′, 5′-CACTGCTTCCCGAATGTCTGA-3′.

Immunohistochemistry. Whole spleens, livers, and kidneys were embedded in Tissue-Tek (Sankura Torrance) and sliced into 5-μm-thick section. Sections were applied to poly lysine–coated slides and fixed in acetone. Spleen and liver sections were then stained with FITC-conjugated anti-B220 or biotin-conjugated anti-Thy1.2 followed by alkaline phosphatase-conjugated anti-FITC and horseradish peroxidase–conjugated streptavidin (Sigma-Aldrich). Fast Blue BB and 3-aminoethylcarbazole (Sigma-Aldrich) solution were added for color development. Kidney sections were stained with FITC–anti–mouse IgG (BD Biosciences).

Autoantibody detection. Anti–double-stranded DNA antibodies were detected using ELISA. 96-well plates were coated with 2.5 $\mu g\ ml^{-1}$ calf thymus DNA in Reacti-bind DNA coating solution (Pierce Chemical Co.). Anti-nuclear antibodies were detected using slides of Hep-2 cells adhered to slides from Antibodies Inc.

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