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Th2 Lymphoproliferative Disorder of Lat<sup>Y136F</sup> Mutant Mice Unfolds Independently of TCR-MHC Engagement and Is Insensitive to the Action of Foxp3<sup>+</sup> Regulatory T Cells<sup>1</sup>

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Mutant mice where tyrosine 136 of linker for activation of T cells (LAT) was replaced with a phenylalanine (Lat<sup>Y136F</sup> mice) develop a fast-onset lymphoproliferative disorder involving polyclonal CD4 T cells that produce massive amounts of Th2 cytokines and trigger severe inflammation and autoantibodies. We analyzed whether the Lat<sup>Y136F</sup> pathology constitutes a bona fide autoimmune disorder dependent on TCR specificity. Using adoptive transfer experiments, we demonstrated that the expansion and uncontrolled Th2-effector function of Lat<sup>Y136F</sup> CD4 cells are not triggered by an MHC class II-driven, autoreactive process. Using Foxp3EGFP reporter mice, we further showed that nonfunctional Foxp3<sup>+</sup> regulatory T cells are present in Lat<sup>Y136F</sup> mice and that pathogenic Lat<sup>Y136F</sup> CD4 T cells were capable of escaping the control of infused wild-type Foxp3<sup>+</sup> regulatory T cells. These results argue against a scenario where the Lat<sup>Y136F</sup> pathology is primarily due to a lack of functional Foxp3<sup>+</sup> regulatory T cells and suggest that a defect intrinsic to Lat<sup>Y136F</sup> CD4 T cells leads to a state of TCR-independent hyperactivity. This abnormal status confers Lat<sup>Y136F</sup> CD4 T cells with the ability to trigger the production of Abs and of autoantibodies in a TCR-independent, quasi-mitogenic fashion. Therefore, despite the presence of autoantibodies causative of severe systemic disease, the pathological conditions observed in Lat<sup>Y136F</sup> mice unfold in an Ag-independent manner and thus do not qualify as a genuine autoimmune disorder. The Journal of Immunology, 2008, 180: 1565–1575.

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inker for activation of T cells (LAT)<sup>4</sup> is a transmembrane adaptor protein that assembles a multiprotein signaling complex through tyrosine-based motifs and links the TCR to intracellular pathways that regulate T cell development and function (for review, see Ref. 1). Analysis of T cells deprived of LAT suggested that it acts primarily as a positive regulator of TCR signaling. Mutant mice where tyrosine 136 of LAT was replaced with a phenylalanine (Lat<sup>Y136F</sup> mice) exhibit a partial developmental block at the two checkpoints that punctuate intrathymic αβ T cell development (1, 2). As a consequence, in 2-wk-old Lat<sup>Y136F</sup> mice, there were eight times fewer CD4 T cells in lymph nodes than in the wild-type (WT) control (3). These αβ<sup>+</sup> CD4 T cells had already acquired an effector Th2 phenotype and embark in an uncontrolled polyclonal proliferation, leading to a 5-fold increase of CD4 T cells in secondary lymphoid organs of 6-week-old Lat<sup>Y136F</sup> mice as compared with the WT control. Owing to the stable Th2 effector phenotype of the expanding CD4 T cells, a massive polyclonal B cell activation ensued, resulting in hypergammaglobulinemia G1 (100-fold) and E (225,000-fold), as well as the production of autoantibodies (2, 3). Two- to 3-mo-old Lat<sup>Y136F</sup> mice showed dense bronchovascular infiltrates and autoantibody deposits in the glomeruli of kidney that are associated with nephritis and proteinuria (3). Therefore, LAT has dual functions in that it acts as a positive regulator of T cell development and also exerts a negative control on the homeostasis of peripheral T cells and on their differentiation into effector cells.

The polyclonal CD4 T cells that successfully mature in Lat<sup>Y136F</sup> mice are selected on and restricted by MHCIi molecules (1). It has been hypothesized that the Lat<sup>Y136F</sup> mutation results in a failure to eliminate self-reactive T cells during intrathymic negative selection (4). According to that view, peripheral Lat<sup>Y136F</sup> CD4 T cells are thought to express autoreactive TCRs that react with self-peptides bound to MHCIi molecules. Following engagement of these putative autoreactive TCRs, the lack of tyrosine 136 of LAT might create an imbalance among activating and inhibitory signaling pathways within individual CD4 T cells and trigger a feed-forward...
activation process resulting in the uncontrolled CD4 T cell differentiation and proliferation that characterize these mice. It has also been suggested that the presence of abnormal CD4 CD25 regulatory T cells in LatY136F mice contributes to the development of the lymphoproliferative disorder (5). Based on the analysis of LatY136F mice expressing an MHCI-restricted TCR originally calibrated in a LAT-sufficient context, we have suggested that the decreased signals expected to emanate from the TCR-LATY136F signaling axis likely support the selection of only those CD4 CD8 thymocytes expressing TCR whose affinity for self is shifted toward higher values than in normal LAT-proficient background (6). However, due to the presence of crippled LATY136F molecules, this increase in TCR affinity is likely cancelled out by a commensurate decrease in TCR output, and the TCR expressed by the CD4 T cells emerging in the LatY136F periphery should not display a strong autoreactivity.

Therefore, the available data leave open the issue of whether the LatY136F disorder constitutes a bona fide autoimmune pathology resulting from a defect in central tolerance and/or from the lack of regulatory T cells. The present study aims at elucidating this issue by identifying the triggers required for the unfolding of the lymphoproliferative disorder in the periphery of LatY136F mice. Using adoptive transfer experiments, we showed that the LatY136F disorder develops in the host periphery in the absence of TCR-MHCI interactions and depends on the presence of IL-7. Therefore, both the expansion and Th2-effecter function of LatY136F CD4 cells do not result from an MHCI-driven, autoreactive process, a finding consistent with the fact that LatY136F CD4 T cells are largely refractory to TCR engagement (2, 7). Moreover, using Foxp3EGFP reporter mice, we revisited the involvement of regulatory T cells in the LatY136F disorder. We showed that CD25 Foxp3 regulatory T cells exist in the periphery of LatY136F mice and are not functional. The TCR-independent proliferation manifested by LatY136F CD4 T cells can, however, escape the control of infused WT Foxp3 regulatory T cells. These results argue against a scenario where the LatY136F pathology is primarily due to a lack of functional Foxp3 regulatory T cells and suggest that a defect intrinsic to LatY136F CD4 T cells accounts for the unfolding of the lymphoproliferative disorder.

**Materials and Methods**

**Mice**

Mice were housed under specific pathogen-free conditions and handled in accordance with French and European directives. Unless specified otherwise, analyses were performed on 5- to 6-wk-old mice. Rag-2−/− (8), Cd3eΔVAV (9), and LatY136F (1) mice have been described. Rag-2−/− mice deficient in IL-7 have been described (10). Cd3eΔVAV mice deficient in MHC class II molecules (Cd3eΔVAV/MHCIγ2a) mice have been described (11). All mice were on a C57BL/6 background.

**Generation of Foxp3EGFP mice**

A C57BL/6/DAC clone denoted RP23-54C14, and containing the mouse Foxp3 gene, was obtained from the Deutsches Ressourcenzentrum für Genomforschung (www.rzd.de). A 7.7-kb targeting vector backbone encompassing exons 8–11 and the 3′ untranslated region of the Foxp3 gene was first subcloned using ET recombination (12). The targeting vector backbone was next abutted with an HSVG-thymidine kinase cassette. An IRRESSGFPCre-neo’ cassette (13) was subsequently inserted through ET recombination into the 3′ untranslated region of the Foxp3 gene, 361 bp downstream of the stop codon. Finally, the targeting vector was linearized by NotI before embryonic stem (ES) cell electroporation. After electroporation of Bruce4 C57BL/6 ES cells (14) and selection in G418 and gancyclovir, colonies were screened for homologous recombination by PCR and by Southern blot analysis with 5′ and 3′ single-copy probes, and with a neomycin probe. The 5′ single-copy probe corresponded to a 493-bp XhoI-PstI fragment isolated from a Foxp3 genomic subclone. When tested on Xhol-digested DNA, it hybridized either to a 7.95-kb WT fragment or to a 6.4-kb recombinant fragment characteristic of the targeted Foxp3EGFP allele. The 3′ single-copy probe corresponded to a 1.18-kb PstI-EcoRI fragment isolated from a Foxp3 genomic subclone. When tested on HindIII-digested DNA, it hybridized either to a 12.5-kb WT fragment or to a 10.5-kb recombinant fragment proper to the targeted Foxp3EGFP allele. Finally, a neo probe was used to ensure that adventitious nonhomologous recombination events had not occurred in the selected clones. Because the Bruce4 ES cells are of male origin, correctly targeted clones were hemizygous for the Foxp3EGFP allele. Mutant ES cells were injected into FVB blastocysts. Screening of mice for the presence of the IRESEGFP insertion was performed by PCR using the following oligonucleotides: Foxp3-001 5′-GCC AAT CCC AGC CTA GCC CCT AGT-3′, Foxp3-002 5′-CAG CTG ACC ACA GTA TAT TGG ATG-3′, EGFP 5′-CAA GTC CGC CAT GCC CGA AGG CTA CG-3′. The primer pair specific for the Foxp3 gene (Foxp3-001 and Foxp3-002) amplifies a 650-bp fragment, and the primer pair specific for the Foxp3EGFP allele (Foxp3-002 and EGFP) amplifies a 1-kb fragment.

**Cell isolation**

CD4 T cells were purified from lymph nodes and spleens from WT and LatY136F mice using a CD4 magnetic microbeads labeling kit and an autoMACS magnetic cell sorter (Miltenyi Biotech). The resulting preparations contained >95% CD4 T cells. CD4 CD25 T cells and CD4 CD25 T cells were isolated from CD45.1 WT mice, whereas CD4 EGFP regulatory T cells were isolated from CD45.2 Foxp3EGFP mice. To isolate CD4 EGFP, CD4 CD25, or CD4 CD25 fractions using a FACSVantage cell sorter (BD Biosciences), CD4 T cells were first enriched using a kit permitting to eliminate CD4 cells (Dynal Biotech).
Adoptive transfer of CD4 T cells

CD4 T cells (3 x 10^6 in 150 µl) were injected i.v. into recipient mice. Spleen and lymph nodes were recovered at various times after transfer. When indicated, purified CD4 T cells were labeled with CFSE (Molecular Probes, Invitrogen) before injection. Spleen and lymph nodes were recovered and CFSE content was analyzed after gating on CD4^+ CD5^+ T cells.

Neonatal transfer of T cells

CD4^+ EGFP^+ or CD4^+ EGFP^+ T cells isolated from CD45.2^+ Foxp3EGFP mice were injected i.p. into 2- to 4-day-old LatY136F neonates (3 x 10^6 cells in a final volume of 20 µl). Mice were bled 4 wk after injection to monitor the extent of transfer, and they were then analyzed at 8 wk of age.

Mixed bone marrow chimeras

Six-week-old Rag-2^-/- mice were irradiated (600 rad) and injected i.v. the next day with 3 x 10^6 bone marrow cells isolated from WT (CD45.1^+) or LatY136F^+ (CD45.2^+) mice and depleted of mature T and B cells before injection. Rag-2^-/- mice were also reconstituted with bone marrow cells from WT and LatY136F mice mixed in a 1:1 ratio. Mice were analyzed 10 wk later.

Abs and flow cytometry

Before staining, cell suspensions were preincubated at 4°C for at least 10 min with the Ab 2,4G2 to block Fc receptors. Single-cell suspensions were stained for 30 min with combinations of FITC-, PE-, PerC-Cy5.5, and APC-, PE-Cy7-, or APC-cy7-conjugated Abs (BD Pharmingen and eBioscience). For Foxp3 intracellular staining, cells were first labeled with Abs directed against surface markers, fixed, and permeabilized with Cytofix/Cytoperm buffer (BD Pharmingen) for 20 min at 10°C. Cells were washed twice with Perm/Wash buffer (BD Pharmingen) and stained with anti-Foxp3-PE Ab (eBioscience) for 30 min. Flow cytometric analysis was performed on an LSRI system or a BD FACS Canto II flow cytometry (BD Biosciences). Acquisition was performed with BD FACSDiva software (BD Biosciences) and analysis with a FlowJo (Tree Star) software.

Determination of serum IgG1 and IgE concentration

The concentration of IgG1 and IgE were determined as described (15).

RESULTS

The LatY136F disorder is T cell autonomous

A total of 3 x 10^6 CD4 T cells purified from WT mice were labeled with CFSE (an intracytoplasmic dye whose intracellular amount is halved at each cell division) and transferred i.v. into histocompatible, CD3e-deficient (Cd3e^A^/A^) mice. Cd3e^A^/A^ hosts are deprived of T cells but harbor B cells (9) and can therefore aid in assessing the impact the transferred T cells have on B cells. As previously described, two coincident and independent phenomena occurred following transfer of WT CD4 T cells into constitutively immunodeficient mice, including Cd3e^A^/A^ mice (11, 16, 17). The vast majority of transferred CD4 T cells proliferated very slowly and retained a naive phenotype, which accounts for the CFSE^+ peaks observed 6 days after transfer (Fig. 1A and data not shown). In contrast, a small proportion of injected WT CD4 T cells proliferated in an explosive manner, yielding the prominent peak of CFSE^- cells observed 6 days after transfer (Fig. 1A). These CFSE^- CD4^+ cells displayed a memory-effector phenotype (Fig. 2, B and C), and their generation has been shown to be independent of IL-7 but strictly dependent on interactions with MHCI molecules (11, 16, 17). It has been further suggested that antigenic peptides derived from enteric bacteria present in constitutively immunodeficient hosts trigger this fast proliferation (17). The fast and slow proliferation displayed by WT CD4 cells does not fully restore the peripheral T cell compartment of Cd3e^A^/A^ hosts (18). For instance, 8 wk after transfer, 6.4 x 10^6 CD4 T cells were recovered on average per spleen, and their impact on endogenous B cells was limited to the normalization of the levels of serum Ig to values close to those found in Cd3e^-sufficient mice (Fig. 2A and data not shown).

Distinct CFSE profiles were obtained after transfer of LatY136F CD4 cells into Cd3e^A^/A^ hosts. Six days after transfer, a rapidly dividing population was not observed, and most LatY136F CD4
cells only proliferated slowly (Fig. 1A). However, in contrast to transferred WT CD4 T cells, this slow proliferation continued unabated, and the CFSE$^+$ peaks observed at day 6 after transfer progressively converted over the following weeks into a single CFSE$^-$ peak (Fig. 3A). Splenomegaly and lymphadenopathy ensued, and 8 wk after transfer the number of LatY136F CD4 T cells reached $\sim 8.3 \times 10^6$ per spleen (Fig. 2). Therefore, the magnitude of the proliferation observed upon adoptive transfer of LatY136F CD4 T cells was 13-fold higher compared with the proliferation observed when WT CD4 T cells were transferred into $\text{Cd3e}^{\Delta\Delta\Delta}$ hosts. The phenotype of LatY136F CD4 cells did not change following their expansion into $\text{Cd3e}^{\Delta\Delta\Delta}$ hosts. They conserved an effector phenotype and continued to express low levels of TCR at their surface (Fig. 2, B and C). Moreover, the expanding LatY136F CD4 T cells had a dramatic effect on the resident B cells, massively inducing their proliferation and triggering IgG1 and IgE hypergammaglobulinemia (Figs. 2A and 3B). Before adoptive transfer, IgE and IgG1 were present at very low concentrations in the serum of $\text{Cd3e}^{\Delta\Delta\Delta}$ mice. Eight weeks after transfer of LatY136F CD4 T cells, serum levels of IgG1 and IgE reached concentrations of 35.4 mg/ml and 846.1 $\mu$g/ml, respectively (Fig. 2). This corresponds to an 84-fold and an 8460-fold elevation of IgG1 and IgE concentrations, respectively, as observed in $\text{Cd3e}^{\Delta\Delta\Delta}$ hosts (Fig. 3B). LatY136F CD4 T cells have an effector phenotype and thus differ from the CD4 T cells purified from WT mice. To exclude that the different behavior of the adoptively transferred WT and LatY136F CD4 T cells was due to their distinct activation status, WT CD4 T cells were activated and expanded in vitro using anti-CD3 and anti-CD28 Abs before their transfer. Eight weeks after transfer into $\text{Cd3e}^{\Delta\Delta\Delta}$ hosts, the CFSE profiles of WT CD4 T cells lacking the peak corresponding to the fast proliferating cells (Fig. 3A, left panels). In contrast, comparable CFSE profiles were observed upon transfer of LatY136F CD4 T cells into $\text{Cd3e}^{\Delta\Delta\Delta}$ hosts that were MHCII-sufficient or -deficient. In both instances, the CFSE profiles of WT CD4 T cells reached $\sim 10^6$ per spleen (Fig. 2). Therefore, the absence of MHCII molecules does not serve in regulating the proliferation of WT CD4 T cells. Therefore, only LatY136F CD4 T cells were capable of recapitulating the pathological features observed in unmanipulated LatY136F mice when transferred into $\text{Cd3e}^{\Delta\Delta\Delta}$ hosts.

The LatY136F peripheral disorder unfolds in the absence of MHCII molecules

To assess whether MHCII molecules play a role in the lymphoproliferative disorder that develops in the periphery of LatY136F mice, we performed adoptive transfer experiments into $\text{Cd3e}^{\Delta\Delta\Delta}$ hosts that expressed neither conventional nor mixed isotype (A$\text{E}_1$) MHCII molecules (11, 20). After transfer into $\text{Cd3e}^{\Delta\Delta\Delta}$ hosts, the CFSE profiles of WT CD4 T cells differed from those observed in MHCII-sufficient hosts in that they lacked the peak corresponding to the fast proliferating cells (Fig. 3, A and B, left panels). In contrast, comparable CFSE profiles were observed upon transfer of LatY136F CD4 T cells into $\text{Cd3e}^{\Delta\Delta\Delta}$ hosts that were MHCII-sufficient or -deficient. In both instances, the CFSE$^+$ peaks observed 6 days after transfer progressively converted into a CFSE$^-$ peak over the following days (Fig. 3, right panels). Therefore, the absence of MHCII molecules does not
 affect the characteristic slow but continuous proliferation of transferred Lat^{Y136F} CD4 T cells. Absolute numbers of CD4 T cells were also assessed at later time points after transfer to take into account both cell proliferation and cell survival. Eight weeks following transfer, 147.5 ± 34.3 × 10^6 (n = 10) and 60.4 ± 14.4 × 10^6 (n = 6) Lat^{Y136F} CD4 T cells were recovered on average per Cd3e^±/± and Cd3e^±/± MHCII^±/± spleen, respectively. Note that 8 wk after transfer into Cd3e^±/± and Cd3e^±/± MHCII^±/± hosts, WT CD4 T cells yielded on average 6.4 ± 1.3 × 10^6 (n = 10) and 0.8 ± 0.2 × 10^6 (n = 6) cells per spleen, respectively. Therefore, Lat^{Y136F} CD4 T cells expanded approximately 2-fold less when transferred into MHCI-deficient hosts than in MHCI-sufficient hosts, and 75-fold more than WT CD4 T cells transferred into MHCII-deficient hosts. Similar results were obtained upon transfer of Lat^{Y136F} CD4 T cells into Cd3e^±/± hosts that were deficient for both MHCI and MHCII molecules (data not shown). MHC molecules are thus dispensable for the slow but sustained proliferation manifested by adoptively transferred Lat^{Y136F} CD4 T cells, and their presence only results in a 2.5-fold enhancement of its magnitude. Therefore, MHCI molecules and, by inference, TCR–MHCII interactions are dispensable for the unfolding of the Lat^{Y136F} pathology.

Preliminary studies using Cd3e^±/±A^±/± hosts in lieu of Cd3e^±/±A^±/± MHCII^±/± hosts showed that adoptive transfer of Lat^{Y136F} CD4 T cells promoted activation of the B cells found in Cd3e^±/±A^±/± hosts (3). However, in Cd3e^±/±A^±/± hosts, the A^±/± chain and the E^±/± chain still associate to form hybrid A^±/±E^±/± MHCII molecules that can be recognized by the transferred CD4 T cells (21, 22). Due to this limitation, we used Cd3e^±/±MHCII^±/± mice in which neither conventional nor mixed isotype MHCII molecules are present on B cells (20) to revisit whether MHCII molecules play a role in the cooperation between Lat^{Y136F} CD4 T cells and B cells. When Cd3e^±/±A^±/± MHCII^±/± mice were injected with Lat^{Y136F} CD4 T cells, increased numbers of B cells and hypergammaglobulinemia G1 and E were observed (Fig. 4B and data not shown). The extent of hypergammaglobulinemia G1 and E was only slightly affected by the lack of MHCII molecules (Fig. 4B). The numbers of activated B cells observed in Cd3e^±/±A^±/± MHCII^±/± mice injected with Lat^{Y136F} CD4 T cells was reduced 1.6-fold, as compared with Cd3e^±/±A^±/± mice injected with Lat^{Y136F} CD4 T cells (Fig. 4B). This may indirectly result from the two-fold reduction in the magnitude of the Lat^{Y136F} CD4 T cell proliferation observed in Cd3e^±/±A^±/± MHCII^±/± mice (Fig. 3). Importantly, activated B cells were all MHCII^±/± and therefore do not originate from the expansion of a few MHCII^±/±, donor-derived B cells that might have adventitiously contaminated the adoptively transferred Lat^{Y136F} CD4 T cells (data not shown). Therefore, based on the use of hosts fully deprived of MHCII molecules, our results confirm that the constitutive T–B cooperation at play in Lat^{Y136F} mice, responsible for hypergammaglobulinemia E and G1, occurs irrespective of the presence of MHCII ligands on B cells.

Proliferation of transferred Lat^{Y136F} CD4 T cells requires IL-7

Several studies have highlighted the role played by IL-7 in the homeostasis of naive and memory CD4 T cells (23–25). Therefore, we asked whether IL-7 is required for the Lat^{Y136F} lymphoproliferative disorder using Rag2−2/−Il-7−/− mice (10)). Nine days after transfer into IL-7-sufficient Rag2−2/− hosts, Lat^{Y136F} CD4 T cells showed a CFSE profile identical with
the one observed upon transfer into \textit{Cd3e}^{5/6\Delta5} hosts (Fig. 1B, right panels). The profile was dominated by slow proliferating cells and already contained a few CFSE\textsuperscript{-} cells. Because \textit{Rag}-2\textsuperscript{−/−} cells differ from \textit{Cd3e}^{5/6\Delta5} hosts by the absence of B cells, this indicates that B cells are not required to trigger the \textit{Lat}^{Y136F} lymphoproliferative disorder. In contrast, following adoptive transfer into \textit{Rag}-2\textsuperscript{−/−}IL-7\textsuperscript{−/−} hosts, \textit{Lat}^{Y136F} CD4 cells showed reduced proliferation (Fig. 1B). Therefore, similar to slowly proliferating, WT CD4 T cells (Fig. 1B, left panels), \textit{Lat}^{Y136F} CD4 T cells expand in an IL-7-dependent manner. Interestingly, the absence of IL-7 had a minimal impact on the survival of \textit{Lat}^{Y136F} CD4 T cells because 9 days after transfer, the numbers of \textit{Lat}^{Y136F} CD4 T cells recovered from \textit{Rag}-2\textsuperscript{−/−}IL-7\textsuperscript{−/−} hosts was only 2.5-fold lower than those recovered from \textit{Rag}-2\textsuperscript{−/−} hosts (data not shown). Therefore, the availability of IL-7 dramatically influenced the ability of \textit{Lat}^{Y136F} CD4 cells to expand following adoptive transfer into lymphopenic hosts, with IL-7 absence having far more negative consequences than the removal of MHCII molecules.

\textbf{Cotransfer of WT CD4 T cells or of Foxp3\textsuperscript{+} regulatory T cells prevents expansion of Lat}^{Y136F} CD4 T cells

Bone marrow cells from WT (CD45.1\textsuperscript{+}) and \textit{Lat}^{Y136F} (CD45.2\textsuperscript{+}) mice were injected into sublethally irradiated \textit{Rag}-2\textsuperscript{−/−} mice, and chimeras were analyzed 10 wk later. The thymus and spleen of \textit{Rag}-2\textsuperscript{−/−} mice that only received WT bone marrow showed a normal complement of T cells (Fig. 4 and data not shown). As expected, thymi of \textit{Rag}-2\textsuperscript{−/−} mice injected with \textit{Lat}^{Y136F} bone marrow cells were hypocellular, and the numbers of T cells found in the spleen were 11.8-fold higher than those found in \textit{Rag}-2\textsuperscript{−/−} mice reconstituted with WT bone marrow cells (Fig. 4). Spleno-megaly resulted from expansion of CD4 Th2 effector cells and of activated B cells (data not shown). Analysis of thymi from \textit{Rag}-2\textsuperscript{−/−} mice reconstituted with a 1:1 mixture of WT and \textit{Lat}^{Y136F} bone marrow cells showed that up to TCR\textequal{}selection, WT and \textit{Lat}^{Y136F}-derived double-negative cells were evenly represented (data not shown). By competing with WT thymocytes for stromal niches (26), and generating fewer double-positive thymocytes (1), \textit{Lat}^{Y136F}-derived, double-negative cells likely account for the approximately three-fold reduced numbers of thymic cells observed in the mixed bone marrow chimeras (Fig. 4). Interestingly, the presence of peripheral, WT CD4 T cells in the mixed bone marrow chimeras prevented the few \textit{Lat}^{Y136F} CD4 T cells that managed to reach the periphery to embark into a proliferative disorder (Fig. 4).

This last observation prompted us to test whether cotransfer of \textit{Lat}^{Y136F} CD4 T cells and of WT CD25\textsuperscript{−}CD4 T cells into \textit{Cd3e}^{5/6\Delta5} hosts would similarly prevent the proliferation of \textit{Lat}^{Y136F} CD4 T cells. Even when mixed at a 1:10 ratio, WT CD25\textsuperscript{−}CD4 T cells readily suppressed the proliferation of the \textit{Lat}^{Y136F} CD4 T cells (Fig. 5, left panels) and their ability to activate endogenous B cells (data not shown). In contrast, when injected at a 1:10 ratio into \textit{Cd3e}^{5/6\Delta5}/MHCII\textsuperscript{−/−} mice, WT CD25\textsuperscript{−}CD4 T cells were unable to prevent the MHCIIN-independent, \textit{Lat}^{Y136F} CD4 T cell proliferation (Fig. 6, right panels). Therefore, the WT CD4 T cells that transiently expand in an explosive and MHCIIN-dependent manner upon transfer into MHCIIN-sufficient \textit{Cd3e}^{5/6\Delta5} hosts (Fig. 3) likely compete for environmental cues and thereby prevent the proliferation of the cotransferred \textit{Lat}^{Y136F} CD4 T cells. A similar reasoning can also apply to the WT CD4 T cells that outnumber the \textit{Lat}^{Y136F}-derived CD4 T cells.
in the periphery of the mixed bone marrow chimeras (Fig. 4). Therefore, in contrast to the “neutral” behavior of endogenous B cells present in Cd3e/H90045/H90045 host, the presence of WT CD4 T cells was capable of controlling the capacity of the pathogenic LatY136F CD4 T cells to expand and activate B cells.

We tested next whether regulatory T cells that express the Foxp3 transcription factor could also control the proliferation of LatY136F CD4 T cells upon cotransfer into Cd3eΔ5Δ5 hosts. Foxp3+ regulatory T cells were purified from WT mice expressing a Foxp3-driven EGFP reporter (see below) and were mixed at a 1:10 ratio with LatY136F CD4 T cells. Following cotransfer into Cd3eΔ5Δ5 hosts, WT Foxp3+ CD4+ regulatory T cells behaved as WT CD4+CD25+ T cells in that they prevented the proliferation of LatY136F CD4 T cells (Fig. 5, left panels). Consistent with the view that regulatory T cells require TCR engagement to exert suppression (27), cotransfer of WT Foxp3+ regulatory T cells and of LatY136F CD4 T cells into Cd3eΔ5Δ5MHCIIdΔ2 hosts clearly demonstrated that the former needed MHCIi molecules to suppress LatY136F CD4 T cell proliferation (Fig. 5).

Presence of Foxp3+ CD4 T cells in LatY136F mice
To determine whether the LatY136F mutation affects the development and function of Foxp3+ regulatory T cells, C57BL/6 knock-in mice expressing an IRESEGFP cassette in the 3' untranslated region of the Foxp3 gene were developed (B6-Foxp3 tm1Mal mice, called Foxp3EGFP mice here; Fig. 6) and crossed to LatY136F mutant mice to generate mice homozygous for the LatY136F mutation and the Foxp3EGFP allele (LatY136F Foxp3EGFP mice). Consistent with results obtained using other Foxp3 reporter mice (28, 29), EGFP expression faithfully marks Foxp3-expressing cells and does not affect the expression of the Foxp3 protein (Fig. 7). Most EGFP+ thymocytes found in WT Foxp3EGFP and LatY136F Foxp3EGFP mice corresponded to CD4 single positive cells and represented 0.22 and 0.19% of total WT Foxp3EGFP and LatY136F Foxp3EGFP thymi, respectively (Fig. 7A). Considering that LatY136F Foxp3EGFP thymi are 2.6-fold smaller than Foxp3EGFP thymi, this corresponds to a 3-fold reduction in their absolute content in EGFP+ regulatory T cells. In contrast to WT Foxp3EGFP thymi in which the vast majority of
T cells purified from To assess whether WT regulatory T cells can control pathogenic organs of T cells is only 1.5-fold reduced in the spleen of CD4 CD3, CD44, CD62L, and CD69 that resemble those found on the are indicated. Also shown are the profiles of WT mice and untreated LatY136F mice. Percentages of CD4 CD5 and CD5 CD4 EGFP CD3 T cells are indicated. Note that the reduction in EGFP fluorescence observed in blood samples is due to the fixation protocol proper to these samples. Numbers of total CD4 T cells present in the spleen and concentrations of serum IgE in the mice specified in the key. WT and LatY136F mice were analyzed at 7 wk of age, and in the case of adoptively transferred neonates, analyses were performed 7 wk after transfer. Also shown are the numbers of EGFP CD4 regulatory T cells (ﬁl) found in the spleen of LatY136F neonates that were adoptively transferred 7 wk before with EGFP CD4 regulatory T cells. Values obtained for each mouse were plotted on linear (CD4 T cell numbers) and logarithmic (IgE concentrations) scales, with the mean of the distribution being indicated by a horizontal bar.

EGFP + cells coexpressed CD25, most EGFP + cells found in LatY136F Foxp3EGFP thymi were CD25 low to CD25 low (Fig. 7A). EGFP + CD4 + cells were also present in the secondary lymphoid organs of LatY136F Foxp3EGFP mice, and they expressed levels of Foxp3 transcription factor similar to the EGFP + CD4 + cells found in Foxp3EGFP mice (Fig. 7B). In contrast, the pathogenic CD4 + Th2 effectors that expand in LatY136F mice were EGFP − (Fig. 7B). Considering the cellularity of WT Foxp3EGFP (mean of 87 × 10^6 cells) and LatY136F Foxp3EGFP (mean of 596 × 10^6 cells) spleens, it can be calculated that the absolute numbers of EGFP + regulatory T cells is only 1.5-fold reduced in the spleen of LatY136F Foxp3EGFP mice. The EGFP + cells found in LatY136F Foxp3EGFP spleen were CD25 + and expressed levels of CD3, CD44, CD62L, and CD69 that resemble those found on the CD4 + CD25 + cells that expand in LatY136F mice (Fig. 7C). When tested in vitro for their functional ability, EGFP + CD4 + regulatory T cells purified from LatY136F Foxp3EGFP mice differed from the EGFP + CD4 + regulatory T cells isolated from WT Foxp3EGFP mice in that they were unable to suppress TCR-induced proliferative responses of WT CD4 T cells (data not shown). Therefore, although a slightly reduced number of Foxp3 + CD4 + regulatory T cells can be found in the periphery of LatY136F mice, they are CD25 − and not functional.

LatY136F CD4 cells escape the control of Foxp3 + regulatory T cells

To assess whether WT regulatory T cells can control pathogenic LatY136F CD4 T cells in a more physiological set-up than that offered by adoptive transfer into Cd3εΔ5Δ5 hosts, EGFP + CD4 + regulatory T cells were sorted with FACS from WT Foxp3EGFP mice and injected into 2- to 4-day-old LatY136F neonates. Because almost no endogenous CD4 T cells are present in the periphery of LatY136F neonates (3), this protocol should provide enough time for proliferation and activation of the transferred WT regulatory T cells and allow them to outnumber the first cohort of CD4 T cells that emerges from the thymus. EGFP expression allowed us to monitor the level of regulatory T cell engraftment and showed that 7 wk after neonatal transfer of 3 × 10^5 EGFP + CD4 + regulatory T cells, the spleen of LatY136F mice contained from 1 to 5 × 10^6 EGFP + CD4 + regulatory T cells (Fig. 8A). However, 7 wk after transfer of EGFP + CD4 + regulatory T cells, the spleens of LatY136F mice also contained an average of 142 × 10^6 LatY136F-derived CD4 T cells that triggered coincident B cell activation, splenomegaly, and hypergammaglobulinemia E and G1 (Fig. 8, A and C). Seven-week-old LatY136F that had received 3 × 10^5 EGFP + CD4 + T cells when they were 4 days old as a control and untreated LatY136F mice contained on average 224 × 10^6 and 223 × 10^6 host-derived CD4 T cells, respectively. Therefore, although it does not prevent the unfolding of the disorder, neonatal transfer of WT regulatory T cells into LatY136F mice was capable of reducing the magnitude of the lymphoproliferative disease measured 7 wk after transfer. As such, the blood of 4-wk-old LatY136F mice that had received either EGFP + CD4 + regulatory T cells or EGFP + CD4 + T cells when they were 4 days old contained 0.7 and 35.8% LatY136F-derived CD4 T cells, respectively (Fig. 8B). In comparison, blood from age-matched, untreated LatY136F mice contained 46.9% CD4 T cells. This indicates that in the presence
of Foxp3⁺ CD4⁺ regulatory T cells, the LatY136F lymphoproliferative disorder develops with protracted kinetics. These results show that the LatY136F pathology is not primarily due to a lack of functional Foxp3⁺ regulatory T cells and suggest that a defect intrinsic to LatY136F CD4 T cells accounts for unfolding of the lymphoproliferative disorder.

Discussion

We showed that adoptive transfer of LatY136F CD4 T cells into Cd3εΔ5Δ5 hosts suffices to recapitulate the rapid-onset, Th2 lymphoproliferative disorder that characterizes LatY11011 mutant mice. Upon transfer, LatY136F CD4 T cells have thus kept their Th2 effector property and proliferative potential. Unexpectedly, their expansion and effector functions were found independent of the presence of MHCII molecules, suggesting that, after successfully developing on the basis of TCR–MHCII interactions, the LatY136F CD4 T cells that reach the periphery convert into a state where they are largely “autistic” to TCR-derived inputs. Moreover, the presence of elevated numbers of activated B cells and of hypergammaglobulinemia E and G1 in hosts lacking all MHCII molecules shows that TCR–MHCII interactions are even not required for induction of class-switch recombination and hypergammaglobulinemia E and G1 by LatY136F CD4 T cells. Therefore, LatY136F CD4 T cells help B cells in an MHCII-independent, “quasi-mitogenic” mode that results in the production of autoantibodies among other Abs (3). In contrast, Ab responses are profoundly blunted in MHCII-deficient mice expressing WT CD4 T cells (30).

The spectrum of TCR Vβ usage among CD4 T cells expanding in the absence of MHCII molecules was similar to that found in CD4 T cells expanding in MHCII-sufficient hosts (data not shown). This suggests that in the absence of MHCII molecules there is no contraction of the TCR repertoire indicative of the selection of unique TCR specificities, and it further supports the view that TCR engagement is not required for the LatY136F lymphoproliferative disorder.

LatY136F CD4 T cells can, however, still respond to environmental cues since their expansion is totally dependent on the presence of IL-7. Interestingly, 6 wk after transfer into Cd3εΔ5Δ5 hosts, LatY136F CD4 T cell expansion reached a plateau corresponding to ~80 × 10⁶ cells per spleen (Fig. 2). This suggests that at early time points enough IL-7 is available in Cd3εΔ5Δ5 hosts to promote both the survival and proliferation of the LatY136F CD4 T cells, whereas at later time points, as their numbers increased, IL-7 can become limiting, promoting their survival without further proliferation. However, it remains to be determined whether IL-7 is sufficient to drive the expansion of the LatY136F CD4 T cells, or whether it plays a permissive role by maintaining a favorable metabolic state averting cell death and allowing other receptors to mediate G1 progression and cell proliferation (31). Importantly, LatY136F CD4 T cells showed a rather unique proliferative behavior in that they do not manifest the explosive and transient kinetics of proliferation that characterize CD4 T cells embarking in Ag-driven responses. As soon as they emerge from the thymus or are adaptively transferred into hosts deprived of T cells, LatY136F CD4 T cells receive stimuli that result in a slow but sustained expansion and manifest full-fledged Th2 effector functions. This behavior also differs from WT CD4 T cells undergoing slow homeostatic-induced proliferation (11, 16, 17).

We also showed that LatY136F mice do contain Foxp3⁺ CD4 T cells. As with the Foxp3⁻ CD4 T cells that expand in LatY136F mice, they do not manifest any functional activity upon stimulation with anti-CD3 and anti-CD28 Abs. However, the expression of LATY136F molecules does not trigger their uncontrolled proliferation, and the reason why the lymphoproliferative disorder induced by LatY136F mutation affects conventional CD4⁺ T cells and spares the Foxp3⁻ regulatory T cells as well as the CD8 T cells remains to be determined. Our findings, based on analysis of LatY136F mice expressing a Foxp3EGFP reporter, conflict with a recent report (5) that claimed that regulatory T cells were absent from LatY136F mice. We showed that the majority of the Foxp3⁺ regulatory T cells present in LatY136F mice were CD25⁻. This may account for the negative results of Koonpaew and colleagues, because they primarily looked for the presence of CD4⁺CD25⁺ T cells. We further showed that adoptive transfer of WT Foxp3⁺ regulatory T cells into LatY136F neonates failed to prevent the subsequent development of the pathology, an observation that is also at variance with the results of Koonpaew and colleagues and might result from the fact that they infused WT CD25⁺ T cells instead of WT Foxp3⁺ T cells (5). By measuring the effects of Foxp3⁺ regulatory T cells at different time points after transfer into LatY136F neonates, we noticed, however, that the presence of Foxp3⁺ regulatory T cell slowed down the unfolding of the LatY136F lymphoproliferative disorder, but in no instance was the disorder prevented. LatY136F CD4 T cells are thus either poorly susceptible to the control exerted by Foxp3⁺ regulatory T cells or their continuous production and magnitude of expansion overwhelmed the functional capacity of the fixed numbers of regulatory T cells that were transferred in LatY136F neonates. It may appear paradoxical that upon adoptive transfer into Cd3εΔ5Δ5 hosts, WT Foxp3⁺ regulatory T cells suppressed the proliferation of coincidently transferred LatY136F CD4 T cells, whereas they were incapable of controlling them when infused into LatY136F neonates. However, in preliminary studies, we found that upon sequential adoptive transfers into Cd3εΔ5Δ5 hosts, LatY136F CD4 T cell effectors showed a gradual diminution of their replicative capacity. A similar replicative exhaustion has been observed for effector T cells during persistent Ag stimulation (32, 33). This may explain that Foxp3⁺ regulatory T cells can control the limited proliferative potential of adoptively transferred LatY136F CD4 T cells purified from 4- to 6-wk-old mice but are overwhelmed by the one manifested by LatY136F CD4 T cells emerging from neonatal thymi.

LatY136F mice cannot be cured by neonatal adoptive transfer of Foxp3⁺ regulatory T cells, and thus they differ from Foxp3⁻ deficient mice and from lymphopenic mice reconstituted with a limited repertoire of CD4 T cells (34, 35). Moreover, the survival kinetics and abnormalities that characterize Foxp3⁻ deficient and LatY136F mice are markedly distinct (36, 37). This suggests that features intrinsic to the LatY136F CD4 T cells, as well as features yet to be discovered, contribute to the Th2 lymphoproliferative disorder. Accordingly, the trigger of the LatY136F pathology is likely due to a defect in a cell-intrinsic mechanism that keeps CD4 T cells in check, and the consequences of this defect are further exacerbated by the lack of cell-extrinsic suppressive mechanisms mediated by Foxp3⁺ regulatory T cells. Peripheral CD4 and CD8 T cells with TGF-β-receptor II deficiency trigger a TCR-specific-dependent, autoimmune pathology and manifest a high pathogenic potential even in the presence of WT regulatory T cells (38). Effector T cells from mice deficient in Cbl-b or TRAF6 were also capable of escaping from suppression by Foxp3⁺ regulatory T cells (39). Therefore, the LatY136F CD4 T cells constitute another example of immune effectors that due to an intrinsic signaling abnormality became resistant to the suppressive action of WT Foxp3⁺ regulatory T cells (39). In WT mice, Foxp3⁺ regulatory
T cells keep in check T cells that are reactive to self- and environmental Ags and limit the magnitude of immune responses in vivo through poorly defined mechanisms (40). The mechanisms underlying the resistance of effector T cells expressing Lat136F molecules or deprived of Cbl-b, TRAF6, or TGF-β receptor II are as yet unclear.

The slow but continuous proliferation manifested by Lat136F CD4 T cells contrasts with the explosive proliferation manifested by the CD4 T cells that emerge in the periphery of Cita-4 and Foxp3-deficient mice and that are hyper-responsive to TCR signals (41–44). The autoimmune process and inflammatory pathology that characterize Cita-4 and Foxp3-deficient mice are thought to occur through TCR-mediated recognition of environmental and/or self-Ags. Consistent with that view, withdrawing or narrowing the source of Ags does prevent the Cita-4 and Foxp3 lymphoproliferative disorders (45, 46). In contrast, the Lat136F lymphoproliferative disorder, including the production of autoantibodies, does not involve TCR–MHCII interactions and therefore does not qualify as a TCR-specificity-dependent autoimmune disease. Therefore, it is possible that some pathological conditions coined as “autoimmune” due to the presence of autoantibodies do not result from a failure of central or peripheral T cell tolerance but instead result from a defect in TCR-operated, negative feedback loops that control T cell hyper-reactivity. Therefore, attempting to identify the autoantigens that trigger such types of disorders constitutes a vain task. Along that line, note that Ag-independent mechanisms have been also recently suggested to account for the B cell hyper-reactivity noted in MRL mice (47). In conclusion, the Lat136F mutation might thus constitute a first example of a mutation that by disturbing the complex signaling hierarchy that is intrinsic to T cell activation renders both the proliferation and function of CD4 Th2 cells independent of the control of the TCR.

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References


