Reduced TCR signaling potential impairs negative selection but does not result in autoimmune disease

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Negative selection and regulatory T (T reg) cell development are two thymus-dependent processes necessary for the enforcement of self-tolerance, and both require high-affinity interactions between the T cell receptor (TCR) and self-ligands. However, it remains unclear if they are similarly impacted by alterations in TCR signaling potential. We generated a knock-in allele (6F) of the TCR ζ chain gene encoding a mutant protein lacking signaling capability whose expression is controlled by endogenous ζ regulatory sequences. Although negative selection was defective in 6F/6F mice, leading to the survival of autoreactive T cells, 6F/6F mice did not develop autoimmune disease. We found that 6F/6F mice generated increased numbers of thymus-derived T reg cells. We show that attenuation of TCR signaling potential selectively impacts downstream signaling responses and that this differential effect favors Foxp3 expression and T reg cell lineage commitment. These results identify a potential compensatory pathway for the enforcement of immune tolerance in response to defective negative selection caused by reduced TCR signaling capability.

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Abbreviations used: ES, embryonic stem; ITAM, immunoreceptor tyrosine-based activation motif.

Most autoreactive T cells are prevented from exiting the thymus by a process termed negative selection (also referred to as recessive tolerance or clonal deletion), which results in death by apoptosis of cells that express a TCR that binds with high affinity to self-MHC/self-ligands (Starr et al., 2003; von Boehmer et al., 2003). The importance of negative selection for the preservation of immune tolerance has been demonstrated by the finding that loss of the transcriptional regulator Aire, which controls the expression of tissue-restricted proteins in the thymus, results in the escape of overtly autoreactive T cells and polyglandular autoimmune disease (Liston et al., 2003; DeVoss et al., 2006; Gavanescu et al., 2007; Mathis and Benoist, 2009). A second mechanism for the enforcement of immune tolerance, termed dominant tolerance, involves the generation in the thymus of regulatory T cells (T reg cells) capable of suppressing the activation of autoreactive conventional T cells that fail to undergo negative selection (Wing and Sakaguchi, 2010). A critical function for dominant tolerance in immune surveillance was revealed by the discovery that in the absence of the transcription factor *Foxp3*, which is required for T reg cell maturation and suppressive activity, both mice and humans develop severe multisystem autoimmune disease (Zheng and Rudensky, 2007).

Studies performed in *Foxp3*-deficient mice, in which T reg cell development is defective, but negative selection appears to be intact, suggest that recessive tolerance alone is insufficient to prevent autoimmune disease in the absence of dominant tolerance (Chen et al., 2005; Zheng and Rudensky, 2007). Whether, or to what extent, dominant tolerance is capable of compensating for defects in recessive tolerance

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is less clear due to the lack of experimental models that have unequivocally established that negative selection is defective but T reg cell development and function are not compromised (Aschenbrenner et al., 2007; Daniely et al., 2010).

The signaling events that regulate and presumably differentiate negative selection and T reg cell development remain incompletely defined. Negative selection and T reg cell development are both promoted by, and dependent on, high-affinity TCR–ligand interactions, as well as TCR signals, and they are thought to share common signaling requirements (Jordan et al., 2001; Starr et al., 2003; Apostolou et al., 2002; Carter et al., 2005; Ordoñez-Rueda et al., 2009). Consistent with this idea, mutations of key signaling intermediates downstream of the TCR, including ZAP-70 and LAT, result in defects in both negative selection and T reg cell development (Sakaguchi et al., 2003; Sommers et al., 2005; Siggs et al., 2007; Hsu et al., 2009; Chuck et al., 2010; Tanaka et al., 2010). It was recently reported that attenuation of TCR-proximal signaling by inactivation of the TCRζ chain immunoreceptor tyrosine based activation motifs (ITAMs) in mice results in a selective defect in negative selection that leads to a rapidly progressive, fatal, multi-system autoimmune disease (Holst et al., 2008). However, these findings contrast with previous data indicating that although attenuation of TCR signaling potential impairs negative selection it does not cause autoimmune disease (Shores et al., 1997; Ardouin et al., 1999; Pitcher et al., 2005b). As these results have important conceptual and clinical implications, further investigation aimed at resolving this discrepancy is warranted.

In this study, we generated knock-in mutations in embryonic stem (ES) cells that result in expression of either WT ζ chain (6Y) or a signaling deficient ζ chain (6F) controlled by endogenous ζ gene regulatory sequences. As predicted, based on previous results obtained with similar mouse models, negative selection was impaired in 6F/6F mice, resulting in the survival of self-reactive T cells. However, 6F/6F mice did not develop autoimmune disease, even under lymphopenic conditions. Unexpectedly, we found that T reg cell numbers and T reg cell suppression activity were increased in 6F/6F mice. We show that the increase in T reg cells is necessary to suppress proliferation of 6F/6F T cells and is required to prevent autoimmune disease in 6F/6F→*Rag1*$^{-/-}$ bone marrow chimeras. Analysis of TCR signaling responses in 6F/6F thymocytes and T cells revealed differential effects on downstream pathways, demonstrating that attenuation of proximal TCR signaling during T cell development does not inhibit, and in fact favors, T reg cell development. Collectively, these findings show that although reduction of TCR signaling potential impairs negative selection, T reg cell development is enhanced and immune tolerance is preserved.

RESULTS

Generation and expression of the 6Y and 6F TCRζ knock-in alleles

The 6Y- and 6F-ζ knock-in mutations were generated in ES cells as depicted in Fig. 1 A. The 6Y allele encodes a wild-type ζ chain that contains a C-terminal FLAG epitope tag. The 6F allele encodes a C-terminal Myc epitope–tagged ζ chain in which the codons for the six immunoreceptor tyrosine-based activation motif (ITAM) tyrosines (Y) were mutated to encode phenylalanine (F).

Because each TCR complex contains one ζ chain homodimer, inactivation of the three ζ chain ITAMs results in the loss of six ITAMs per TCR complex. Consequently, thymocytes and T cells in homozygous 6F/6F mice express TCR complexes that contain a total of 4 ITAMs (contributed by the CD3-γε and CD3-δε dimers), whereas thymocytes and T cells in wild-type 6Y/6Y mice express TCRs containing the normal complement of 10 ITAMs (Fig. 1 B).

TCRζ transcript levels were similar in thymocytes and T cells from 6Y/6Y and 6F/6F mice (Fig. 1 C), and ζ chain proteins of the predicted mass (slightly larger than wild-type ζ because of the addition of the epitope tags) were detected by Western blotting with antibody that recognizes both the 6Y and 6Fζ chains (Fig. 1 D). We also confirmed that the 6Y and 6Fζ chains were able to homodimerize and assemble with the multimeric TCR complex (Fig. 1, E and F). Partial tyrosine phosphorylation of 6Yζ (equivalent to wild-type p21ζ) was detected in freshly isolated thymocytes, whereas stimulation with pervanadate resulted in phosphorylation of all six of the 6Yζ ITAM tyrosines (equivalent to wild-type p23ζ; Fig. 1 G). In contrast, and as predicted, because all six ITAM tyrosines are mutated to phenylalanine, tyrosine phosphorylation of 6Fζ was undetectable ex vivo or after pervanadate stimulation (Fig. 1 G). Intracellular staining with antibody that recognizes both 6Yζ and 6Fζ demonstrated similar protein expression in 6Y/6Y and 6F/6F thymocytes at all stages of development (Fig. 1 H). Together, these results confirm that expression of the 6Y and 6F knock-in alleles closely approximates that of the endogenous ζ gene. Finally, intracellular staining with anti-FLAG and anti-Myc mAbs verified that thymocytes and T cells from 6Y/6Y mice express only the 6Yζ chain, whereas thymocytes and T cells from 6F/6F mice express only 6Fζ chain (Fig. 2 A).

T cell development in 6F/6F mice

Surface staining of 6F/6F thymocytes for CD4 and CD8 revealed an essentially normal developmental profile resembling that of control 6Y/6Y thymocytes (Fig. 2 B). However, thymocyte numbers were consistently lower in 6F/6F mice compared with age/gender-matched control 6Y/6Y mice, and this was due to a selective reduction in $CD4^+CD8^-$ ($CD4^+$) and $CD4^-CD8^+$ ($CD8^+$) cells (Fig. 2 C and not depicted). $CD4^+$ and $CD8^+$ peripheral T cells were also significantly reduced in 6F/6F mice relative to 6Y/6Y mice (Fig. 2, B and C). Consistent with data obtained with other 6F (or equivalent) mouse models (Shores et al., 1997; Pitcher et al., 2005b; Holst et al., 2008), positive selection was markedly impaired in 6F/6F mice (unpublished data). Also in agreement with previous results, γδ T cell development (Hayes et al., 2005) and NK T cell development (Becker et al., 2010) were impaired in 6F/6F mice (unpublished data).

TCR surface expression, assessed by staining with anti-TCRβ, was equivalent on CD4+ and CD8+ thymocytes and T cells in 6Y/6Y and 6F/6F mice, but TCR levels were elevated on DP thymocytes in 6F/6F mice (Fig. 2 D). This phenotype is consistent with previous data demonstrating that in DP thymocytes, TCR recycling and ζ degradation are regulated by ζ chain ITAM phosphorylation (Myers et al., 2006). Indeed, when DP thymocytes from 6Y/6Y mice were cultured in vitro in the absence of TCR simulation, which results in the dephosphorylation of ζ ITAMs (Nakayama et al., 1989),

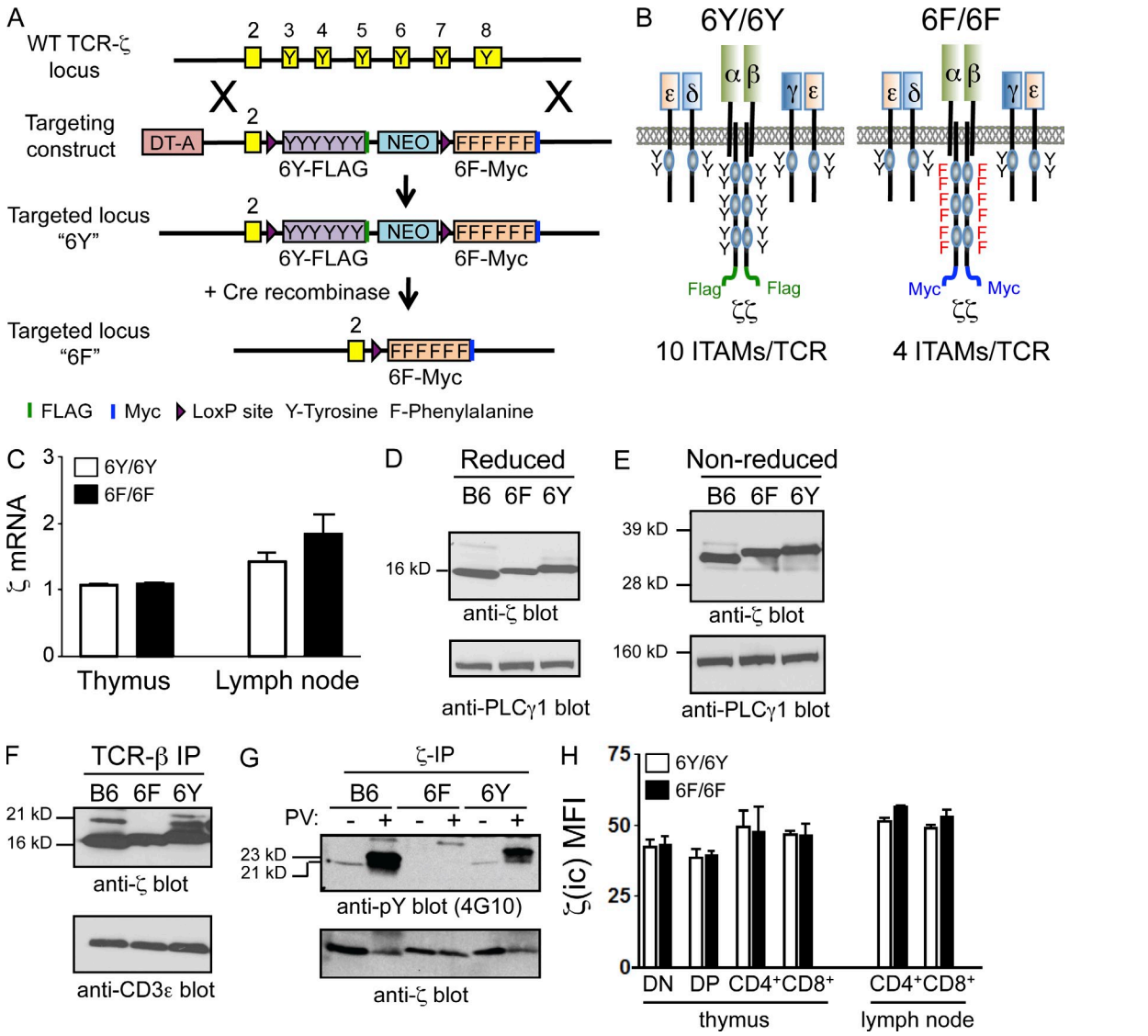

Figure 1. TCRζ chain expression in 6Y/6Y and 6F/6F mice. (A) Outline of targeting strategy. (top) Schematics of the TCRζ locus and the 6Y/6F targeting construct. (middle) Schematic of the 6Y knock-in allele generated by homologous recombination in ES cells. (bottom) Schematic of the 6F knock-in allele generated after Cre-mediated removal of the 6Y-FLAG-*NEO* cassette. (B) Diagram of TCR complexes expressed in 6Y/6Y and 6F/6F mice. (C) Evaluation of TCRζ gene expression in total thymocytes and lymph node T cells from 6Y/6Y ($n = 3$) and 6F/6F ($n = 3$) mice by quantitative real-time PCR. Differences between 6Y/6Y and 6F/6F cohorts were not significant. (D) Thymocyte whole-cell lysates from B6, 6Y/6Y (6Y), and 6F/6F (6F) mice were subjected to reducing SDS-PAGE and blotted with anti-ζ and anti-PLCγ as loading controls. (E) Analysis was identical to that in D except that cell lysates were subjected to nonreducing SDS-PAGE. (F) Thymocyte whole-cell lysates were incubated with anti-TCRβ, and then immunoprecipitated proteins were resolved by reducing SDS-PAGE and blotted with anti-ζ and anti-CD3ε. Positions of the 16- and 21-kD ζ isoforms are shown. (G) Total thymocytes from B6, 6Y/6Y (6Y), and 6F/6F (6F) mice were left unstimulated (−) or stimulated with pervanadate to induce tyrosine phosphorylation of cellular proteins. Whole-cell lysates were incubated with anti-ζ and immunoprecipitated proteins were resolved by reducing SDS-PAGE and blotted with anti-phosphotyrosine antibody (4G10) or anti-ζ. (H) Total thymocytes or total lymph node cells from 6Y/6Y, 6F/6F, and $\zeta^{-/-}$ mice were surface stained with anti-CD4 and anti-CD8, stained intracellularly with anti-ζ, and then analyzed by FACS. Shown are mean fluorescence intensity (MFI) of intracellular ζ protein on gated DN, DP, CD4+, and CD8+ thymocytes or gated CD4+ and CD8+ lymph node T cells after subtraction of MFI in the same population of cells from $\zeta^{-/-}$ mice. Differences between 6Y/6Y and 6F/6F cohorts were not significant. Results shown in C–H are representative of three experiments.

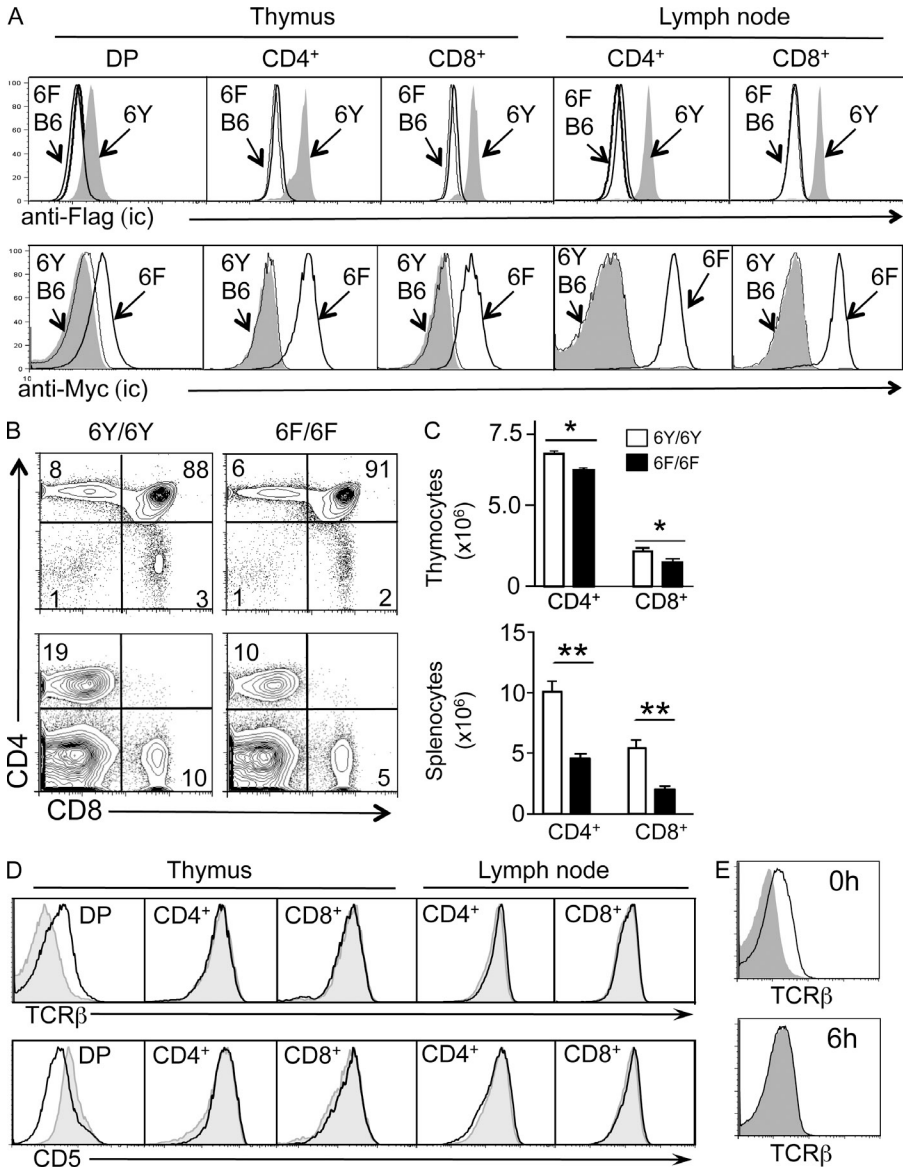

Figure 2. Phenotype of 6Y/6Y and 6F/6F mice. (A) Intracellular staining was performed with anti-FLAG (top) or anti-Myc (bottom) to detect expression of the 6Yζ (FLAG-tagged) and 6Fζ (Myc-tagged) proteins, respectively. B6 mice were included as a negative control for FLAG and Myc intracellular staining. Results are representative of three independent experiments. (B) CD4 versus CD8 profiles of thymocytes (top) and splenocytes (bottom) from 6Y/6Y and 6F/6F mice. Numbers in quadrants are percentage of total cells. Results are representative of 15 mice of each group. (C) Number of $CD4^+$ and $CD8^+$ thymocytes (top) and splenocytes (bottom) in 6Y/6Y and 6F/6F mice. Fifteen 3–4-mo-old mice were analyzed in each group. *, $P < 0.05$; **, $P < 0.01$. Data shown are mean ± SD. (D) TCRβ surface staining (top) and CD5 surface staining (bottom) of thymocytes and lymph node T cells from 6Y/6Y mice (shaded) and 6F/6F mice (black line). 1 experiment, representative of 15. (E) Thymocytes from 6Y/6Y mice (shaded) and 6F/6F mice (black line) were maintained at 4°C (0 h) or cultured at 37°C for 6 h, and then stained for TCR surface expression with anti-TCRβ. One representative of seven experiments.

TCR surface expression increased to levels similar to those on ex vivo DP thymocytes from 6F/6F mice (Fig. 2 E). Notably, even though TCR surface expression was increased on DP thymocytes in 6F/6F mice, CD5 surface expression was reduced (Fig. 2 D), reflecting the reduced signaling potential of 6Fζ-associated TCRs (Azzam et al., 1998).

Defective negative selection in the absence of ζ ITAMs

Previous work has shown that negative selection is defective in $\zeta^{-/-}$ mice that express a transgenic 6F (or equivalent) ζ chain in lieu of wild-type ζ chain (Ardouin et al., 1999; Holst et al., 2008; Pitcher et al., 2005a; Shores et al., 1997). To determine if negative selection is affected in 6F/6F mice, we first evaluated TCR-induced apoptosis in DP thymocytes during in vitro culture. Notably, DP thymocytes from 6F/6F mice were resistant to anti-CD3 + anti-CD28–induced apoptosis over a range of stimulating conditions, revealing a quantitative defect in TCR-induced negative selection (Fig. 3 A). Negative selection of H-Y TCR transgenic thymocytes in response to ubiquitous male antigen was also impaired, but not abrogated, by the 6F mutation (Fig. 3 B). Interestingly, Mtv-8 and Mtv-9 superantigen-mediated deletion of $V_\beta5^+$, $V_\beta11^+$, and $V_\beta12^+$ SP thymocytes, which requires expression of the MHC molecule, I–E (Kappler et al., 1987; Irwin and Gascoigne, 1993) was unaffected or only slightly attenuated in I-E^+ (H-$2^{b/d}$) 6F/6F mice, indicating that the impact of ζ ITAM inactivation on negative selection varies depending on the nature of the negatively selecting interaction (Fig. 3 C).

To evaluate the effect of inactivating ζ chain ITAMs on TCR signaling pathways known to be important for negative selection, DP thymocytes from 6Y/6Y or 6F/6F mice were stimulated with anti-TCRβ and anti-CD4, and the activation of downstream effectors was examined. Phosphorylation of ZAP-70, Cbl, and ERK was reduced in 6F/6F DP thymocytes after TCR/CD4 cross-linking (Fig. 4 A). In contrast, TCR-induced calcium mobilization was equivalent in 6Y/6Y and 6F/6F DP and $CD4^+$ thymocytes, even with submaximal concentrations of stimulating antibody (Fig. 4 B). Jnk and p38 have been implicated in negative selection

(Rincón et al., 1998; Sugawara et al., 1998); yet, surprisingly, phosphorylation of Jnk and p38 was also unaffected in 6F/6F thymocytes (Fig. 4 A). However, TCR-induced phosphorylation of ERK5 was reduced in 6F/6F thymocytes (Fig. 4 A). ERK5 is required for the induction/activation of the orphan steroid receptor family member Nur77 (Sohn et al., 2007), and Nur77 in turn has been implicated in negative selection through its ability to convert Bcl-2 into a proapoptotic molecule (Sohn et al., 2007). As shown in Fig. 4 C, Nur77 induction was also impaired in 6F/6F thymocytes after TCR cross-linking. This result is consistent

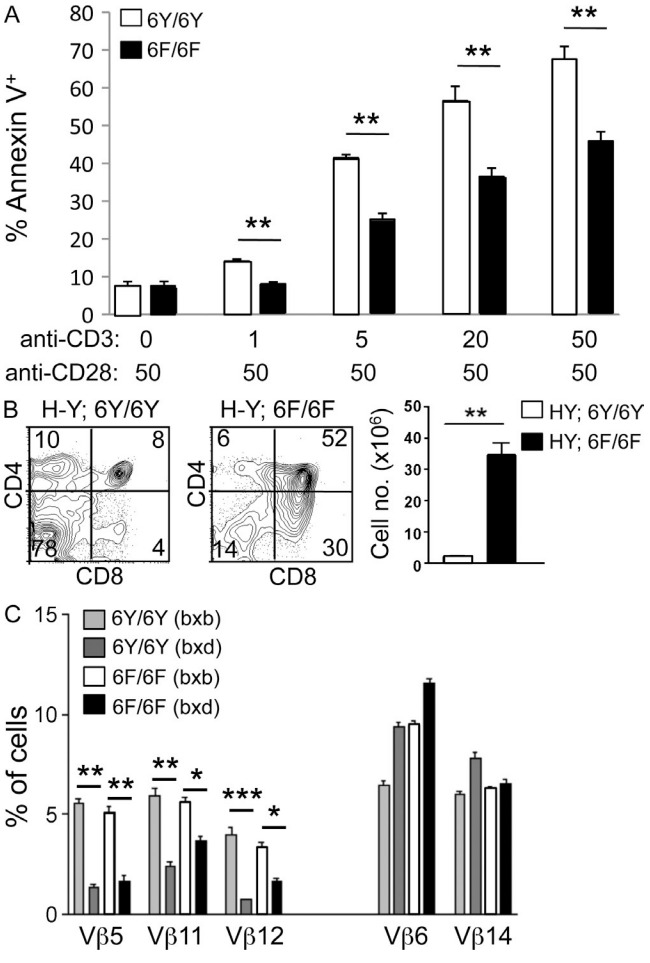

Figure 3. Defective negative selection in 6F/6F mice. (A) Thymocytes from 6Y/6Y and 6F/6F mice were stimulated with anti-CD3 and anti-CD28 for 24 h. Cell death was assessed by surface staining for CD4, CD8, and Annexin V and analysis of gated DP thymocytes by FACS. Data shown are mean ± SD from three experiments. **, $P < 0.01$. (B) CD4 versus CD8 staining of thymocytes from H-Y TCR transgenic 6Y/6Y (left) and 6F/6F (middle) male mice. 1 representative experiment of 13. Right panel shows number of thymocytes in H-Y TCR transgenic 6Y/6Y and 6F/6F male mice. Data shown are mean ± SD **, $P < 0.01$; $n = 13$ each. (C) Percentage of Mtv-8,9 superantigen-deleted (Vβ5+, Vβ11+ or Vβ12+) or non–superantigen-deleted (Vβ6 and Vβ14) CD4+ thymocytes in H-$2^{b/b}$ and H-$2^{b/d}$ 6Y/6Y and 6F/6F mice. Six mice were analyzed in each group. Data shown are mean ± SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

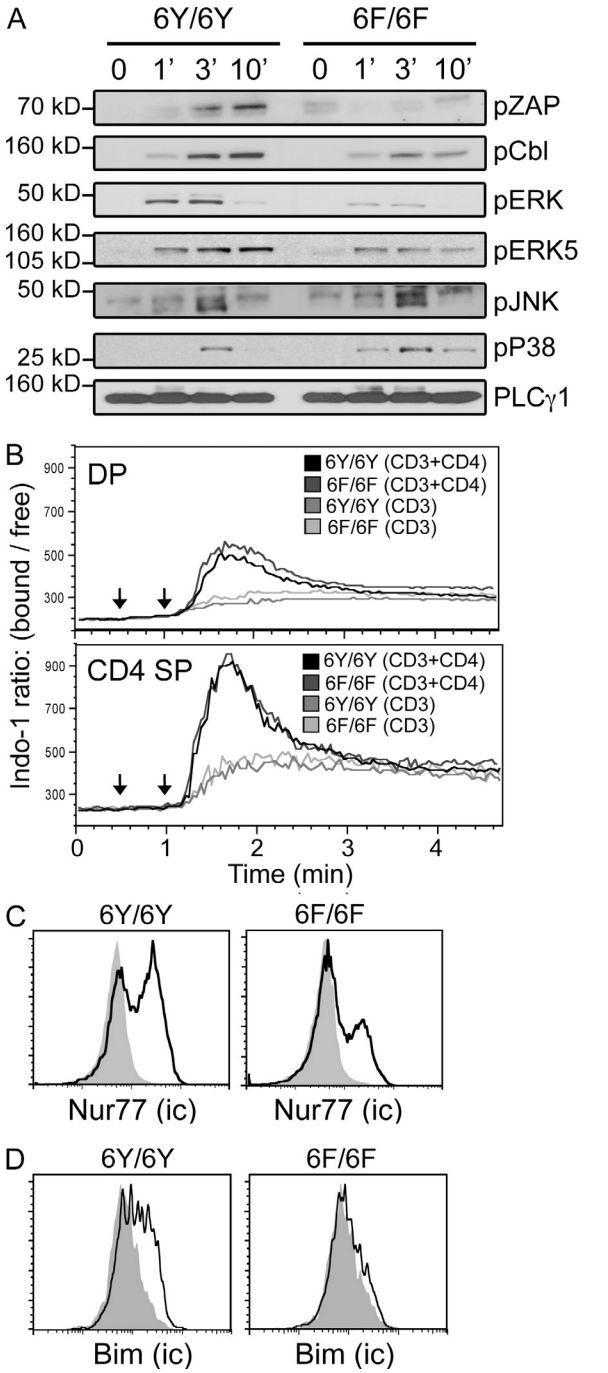

Figure 4. TCR signaling responses in 6F/6F mice. (A) DP thymocytes from 6Y/6Y and 6F/6F mice were stimulated with anti-CD3 and anti-CD4 for the indicated times, and whole-cell extracts were analyzed by SDS-PAGE and Western blotting with the indicated antibodies. One representative of three experiments. (B) Calcium flux in 6Y/6Y or 6F/6F thymocytes induced by stimulation with anti-CD3 (10 µg) or anti-CD3 (10 µg) + anti-CD4 (7.5 µg). First arrow indicates time when biotinylated stimulating antibody was added; second arrow indicates time of antibody cross-linking by addition of avidin. One representative of three experiments. (C and D), intracellular staining for Nur77 (C) or Bim (D) in CD4+ thymocytes after in vitro culture for 6 h (Nur77) or 4 h (Bim) without stimulation (gray shaded) or with anti-CD3 plus anti-CD28 stimulation (black line). One representative of three experiments.

with recent data demonstrating that the level of Nur77 induction directly reflects TCR signal intensity (Moran et al., 2011). TCR-mediated induction of the proapoptotic molecule Bim, which has also been shown to be important for negative selection (Bouillet et al., 2002), was likewise reduced in 6F/6F SP thymocytes (Fig. 4 D). Collectively, these findings suggested that the peripheral T cell repertoire may be skewed in 6F/6F mice toward cells that express TCRs with high affinity to self-ligands that have escaped negative selection.

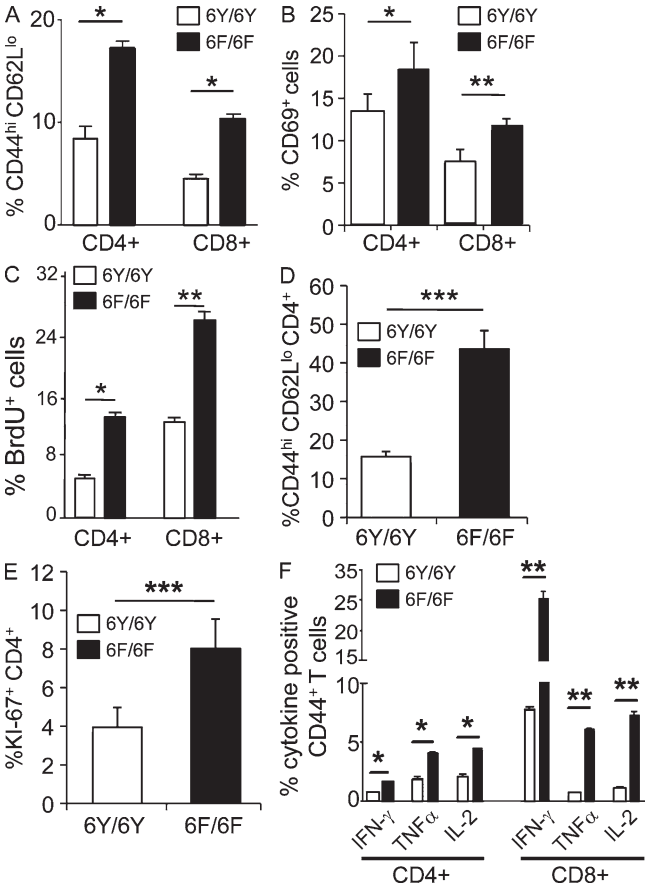

Figure 5. Activated-memory phenotype of T cells in 6F/6F mice. (A) Percentage of ($CD44^{hi}CD62L^{lo}$) $CD4^+$ and $CD8^+$ splenocytes in 6Y/6Y ($n = 8$) and 6F/6F ($n = 8$) mice. (B) Percentage of $CD69^+$ $CD4^+$ and $CD8^+$ splenocytes in 6Y/6Y ($n = 6$) and 6F/6F ($n = 6$) mice. (C) Percentage of proliferating ($BrdU^+$) T cells in 6Y/6Y ($n = 6$) and 6F/6F ($n = 6$) mice after 3 d of BrdU oral administration. (D) Percentage of donor-derived memory-phenotype ($CD44^{hi}CD62L^{lo}$) $CD4^+$ T cells and KI-67^+ $CD4^+$ T cells (E) in sublethally (650 Rads) irradiated B6(CD45.1) recipient mice 6 wk after transfer of a 1:1 mixture of lineage-depleted 6Y/6Y and 6F/6F bone marrow cells. Donor (6Y/6Y or 6F/6F) origin was determined by intracellular staining with anti-Flag and anti-Myc on gated $CD45.2^+$ $CD4^+$ T cells. 4 mice of each group were analyzed. For A–E, data shown are mean ± SD. (F) Intracellular staining of $CD44^{hi}$ $CD4^+$ and $CD44^{hi}$ $CD8^+$ splenocytes from 6Y/6Y ($n = 6$) and 6F/6F ($n = 6$) mice for expression of IFN-γ, TNF, or IL-2. Shown are mean ± SD of percentage of $CD44^{hi}$ T cells positive for the indicated cytokine. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

6F/6F T cells exhibit a phenotype consistent with high self-affinity

A higher percentage of T cells in 6F/6F mice exhibited a memory-like phenotype ($CD62L^{lo}CD44^{hi}$), expressed the cell surface activation marker CD69, and were proliferating in vivo compared with T cells in 6Y/6Y controls (Fig. 5, A–C). Some of these effects could be attributed to lymphopenia-induced expansion, which results in a phenotype that resembles antigen-experienced memory cells (Jameson, 2002; Goldrath et al., 2004). However, 6F/6F T cells also exhibited a similar activated-memory phenotype in a nonlymphopenic environment (Fig. 5, D and E). A higher percentage of $CD44^{hi}$ $CD4^+$ and $CD8^+$ T cells in 6F/6F mice expressed IL-2 and the proinflammatory cytokines IFN-γ and TNF (Fig. 5 F). Similar to what we had observed in $\zeta^{-/-}$ mice (Shores et al., 1998), $CD4^+$ T cells from 6F/6F mice displayed significant Th1 skewing, both ex vivo and after in vitro stimulation (unpublished data).

6F/6F mice do not develop spontaneous autoimmune disease

6F/6F mice were fertile, had normal life spans, and did not display signs of increased morbidity compared with age and gender matched 6Y/6Y mice (unpublished data). Random histological screening of multiple organs from 4–9 mo old mice failed to identify a significant increase in the incidence of inflammatory lesions or lymphocyte infiltrates compared with 6Y/6Y controls (Fig. 6, A and B). Serologic screening for autoantibodies was consistently negative (not depicted) and 6F/6F mice did not contain increased numbers of $IL\text{-}17^+$ (Th17) T cells (Fig. 6 C). $6F/^-$ mice, which contain one ζ-null allele and express lower surface levels of TCR on DP thymocytes (comparable to 6Y/6Y mice; not depicted) also did not develop autoimmune disease, demonstrating that further reduction of TCR signaling potential does not predispose mice to autoimmunity (Fig. 6 A). Although 6F/6F mice did not exhibit signs of spontaneous autoimmunity, we noted a consistent, though not statistically significant, increase in the clinical severity of induced experimental autoimmune encephalomyelitis relative to 6Y/6Y mice (Fig. 6 D).

Increased generation of T reg cells in 6F/6F mice

To explain the absence of autoimmunity in 6F/6F mice, we next examined the development and function of $Foxp3^+$ T reg cells. Interestingly, we found that both the percentage and number of $Foxp3^+$ $CD4^+$ thymocytes and T cells were significantly increased in 6F/6F mice relative to either 6Y/6Y or B6 controls (Fig. 7, A–C). Expression of T reg cell markers, including CD25, was similar on $Foxp3^+$ $CD4^+$ T cells in 6Y/6Y and 6F/6F mice (unpublished data). In addition, we verified that ζ chain expression was equivalent in $Foxp3^+$ $CD4^+$ thymocytes and T cells from 6Y/6Y and 6F/6F mice (unpublished data).

Several findings strongly suggested that the higher number of T reg cells in 6F/6F mice was due, at least in part, to increased generation of thymus-derived T reg cells rather than

A Autoimmune disease in 6Y/6Y, 6F/6F 6Y/- and 6F/- mice

Organ	6Y/6Y	6F/6F	6Y/-	6F/-
salivary gland	0/10	0/10	0/5	0/5
liver	0/10	1/10[b]	0/5	0/5
lung	1/10[a]	0/10	0/5	0/5
kidney	0/10	0/10	0/5	0/5
intestine	0/10	0/10	0/5	0/5

[a,b,] several small perivascular infiltrates.

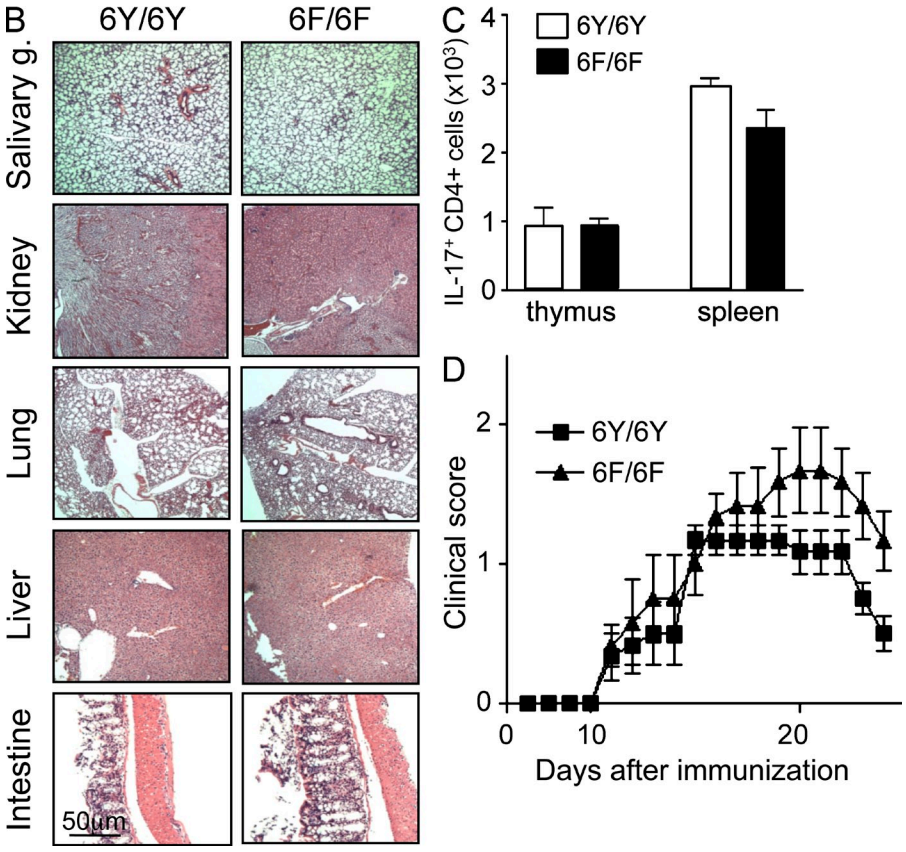

Figure 6. Absence of autoimmune disease in 6F/6F mice. (A) Prevalence of inflammatory lesions in 6-mo-old 6F/6F and 6F/− mice. (B) Representative hematoxylin and eosin stained sections of formalin fixed, paraffin embedded tissues from 6-mo-old 6Y/6Y or 6F/6F mice. Three sections obtained from different regions of the indicated tissues were stained and examined. (C) Percentage of IL-17+ CD4+ thymocytes and T cells in 6Y/6Y and 6F/6F mice detected by intracellular staining. Results are shown as mean ± SD of data from four mice of each group. Differences between 6Y/6Y and 6F/6F cohorts were not significant. (D) Induction of EAE in 6Y/6Y and 6F/6F mice. Mice were immunized with MOG peptide at Day 0, and clinical scores were evaluated daily beginning at day 7. Results shown are mean ± SD from two experiments. 6Y/6Y (7 mice), 6F/6F (6 mice). Differences in clinical scores were not statistically significant.

entirely to peripheral expansion of T reg cells or increased generation of peripheral T reg cells. First, BrdU labeling of Foxp3+ CD4+ cells was comparable in 6Y/6Y and 6F/6F mice (Fig. 7 D). Second, the percentage of Foxp3+ CD4+ thymocytes was significantly increased in 4-d-old 6F/6F mice (Fig. 7 E), when the first wave of T reg cell development can be observed in the thymus and when Foxp3+ T cells are not present in the periphery (Fontenot et al., 2005). Third, the percentage of Foxp3− CD25+ CD4+ thymocytes, which are thought to be the direct progenitors of Foxp3+ T reg cells (Lio and Hsieh, 2008), was markedly elevated in 6F/6F mice (Fig. 7 F). The TCR-Vβ repertoire was similar on Foxp3+ CD4+ thymocytes from 6Y/6Y and 6F/6F mice, indicating that the increase in T reg cells was not caused by clonal expansion (Fig. 7 G). Finally, the percentage of 6F/6F-derived T reg cells was also elevated in nonlymphopenic 6F/6F→B6 bone marrow chimeras, demonstrating that this effect is T cell intrinsic and is not secondary to lymphopenia-induced expansion (unpublished data).

Increased numbers of T reg cells are required for suppression of 6F/6F T cells

To determine if the T reg cell population in 6F/6F mice exhibited normal suppression activity, CD25+ CD4+ T cells from 6Y/6Y or 6F/6F mice, which are enriched for Foxp3+ T reg cells (Wing and Sakaguchi, 2010), were tested for their ability to suppress the proliferation of naive CD25− effector CD4 T cells from B6 mice in a standard in vitro suppression assay. Notably, 6F/6F T reg cells exhibited enhanced suppressive activity relative to either 6Y/6Y or B6 T reg cells, demonstrating that they were fully capable of suppressing the proliferation of effector T cells (Fig. 8 A). We next determined if greater numbers of T reg cells were required to suppress the proliferation of 6F/6F effector T cells. In these experiments, CD25+ CD4+ T cells (T reg cells) from B6 mice were mixed at varying ratios with naive CD25− CD4+ effector T cells from 6Y/6Y, 6F/6F, or B6 mice. As shown in Fig. 8 B, four- to eightfold higher T reg cell/T effector ratios were required to suppress the proliferation of 6F/6F CD4+ effector T cells to the same extent as 6Y/6Y or B6 effectors, demonstrating that increased numbers of T reg cells are necessary for the suppression of 6F/6F effector T cells.

Rag1$^{-/-}$ recipients of 6F/6F bone marrow develop widespread autoimmune disease

The absence of autoimmune disease in 6F/6F mice contrasts with previous results demonstrating that a similar (6F) mutation

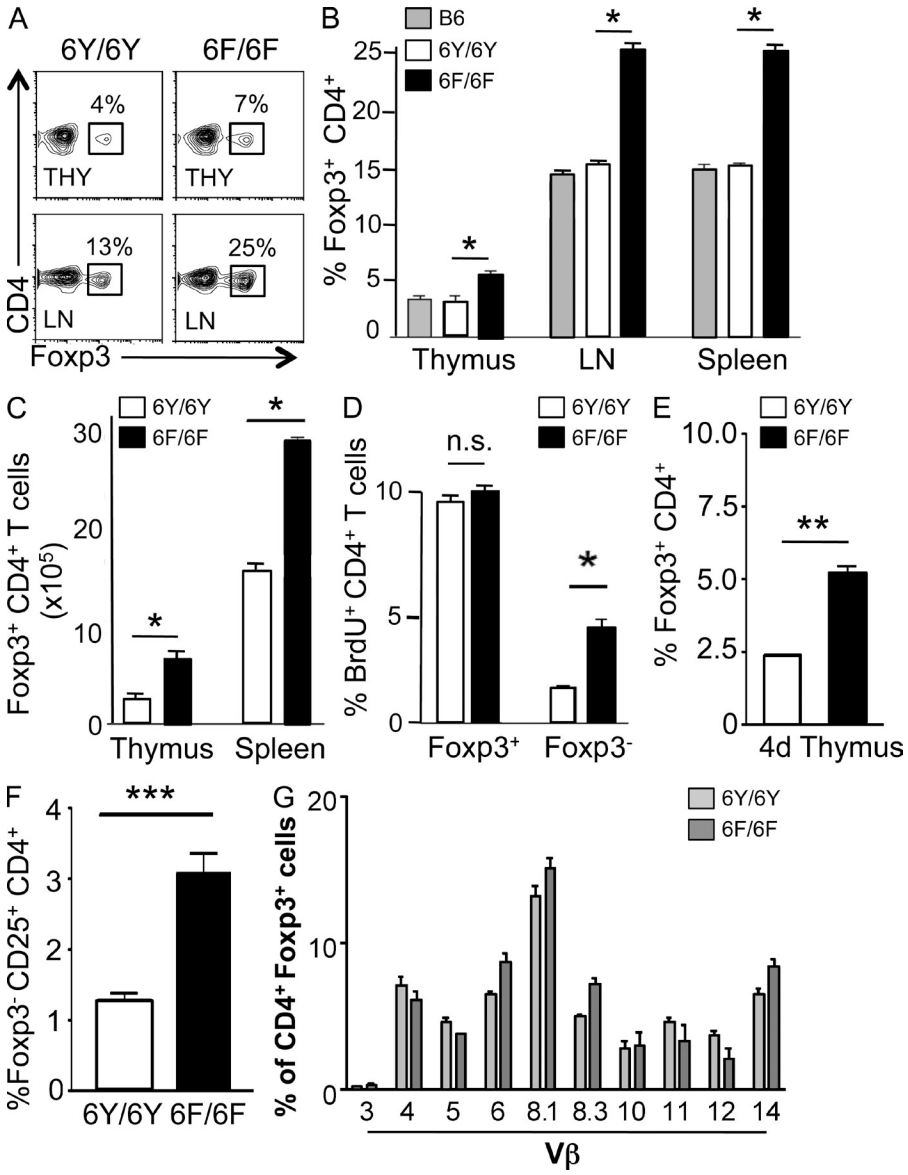

Figure 7. Increased numbers of Foxp3+ CD4+ thymocytes and T cells in 6F/6F mice. (A) CD4 versus Foxp3 plots of gated CD4+ thymocytes (THY) or lymph node (LN) cells from 6Y/6Y and 6F/6F mice. Data shown are 1 representative of 11 mice from each genotype. (B) Percentage of Foxp3+ CD4+ cells in the indicated tissues of B6 ($n = 3$), 6Y/6Y ($n = 11$), or 6F/6F ($n = 11$) mice. (C) Number of Foxp3+ CD4+ cells in the thymus and spleen of 6Y/6Y ($n = 8$) and 6F/6F ($n = 8$) mice. (D) Percentage of proliferating (BrdU+) Foxp3+ CD4+ and Foxp3− CD4+ T cells in 6Y/6Y ($n = 6$) and 6F/6F ($n = 6$) mice after 3 d of BrdU oral administration. (E) Percentage of Foxp3+ CD4+ cells in the thymus of 4-d-old 6Y/6Y ($n = 3$) and 6F/6F ($n = 3$) mice. (F) Percentage of Foxp3- CD25+ CD4+ cells in the thymus of adult 6Y/6Y ($n = 8$) and 6F/6F ($n = 8$) mice. (G) TCRVβ repertoire of Foxp3+ CD4 SP splenocytes in 6Y/6Y ($n = 4$) and 6F/6F ($n = 4$) mice. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$. For all bar graphs, data shown are mean ± SD.

of ζ ITAMs causes lethal multisystem autoimmune disease (Holst et al., 2008). In that study, bone marrow cells from $\zeta^{-/-}$ mice were infected with retrovirus encoding 6Fζ, and then injected into irradiated *Rag1*$^{-/-}$ mice (Holst et al., 2008). Notably, unlike 6F/6F mice, no increase in T reg cells was reported in ζ6F bone marrow recipient *Rag1*$^{-/-}$ hosts (Holst et al., 2008). To determine if the different results obtained could be attributed to the experimental design, we generated bone marrow chimeras by injecting lineage-depleted bone marrow cells from 6Y/6Y or 6F/6F mice into irradiated *Rag1*$^{-/-}$ mice. Analysis of chimeric mice 6 mo after bone marrow transfer revealed severe multi-organ autoimmune disease in all 6F/6F→*Rag1*$^{-/-}$ chimeras, resembling those reported by Holst et al. (2008; Fig. 9, A and B). We also detected rare lymphocyte infiltrates in 6Y/6Y→*Rag1*$^{-/-}$ recipients that were smaller in size and limited to single organs in contrast to the widespread disease in 6F/6F bone marrow recipients (Fig. 9 A). Moreover, antinuclear autoantibodies and increased percentages of IL-17+ CD4+ cells were detected in both 6Y/6Y→*Rag1*$^{-/-}$ and 6F/6F→*Rag1*$^{-/-}$ bone marrow chimeras, suggesting that all *Rag1*$^{-/-}$ bone marrow recipient mice were predisposed to autoimmunity, regardless of the source of donor-transplanted bone marrow cells (Fig. 9, C and D). Irradiated B6 recipients injected with 6F/6F bone marrow cells did not develop autoimmune disease (unpublished data), identifying *Rag1*$^{-/-}$ recipients as a critical factor in autoimmune disease susceptibility. All 6F/6F→*Rag1*$^{-/-}$ chimeric mice were severely lymphopenic; however, lymphopenia alone could not account for their susceptibility to autoimmune disease, as intravenous transfer of T cells from 6F/6F mice into *Rag1*$^{-/-}$ mice failed to elicit autoimmune disease (unpublished data). When analyzed 6 mo after bone marrow transfer, 6F/6F→*Rag1*$^{-/-}$ chimeric mice contained high percentages of Foxp3+ CD4+ cells (similar to 6F/6F donors; unpublished data); however, it was unclear if this was because of lymphopenia-induced expansion (Milner et al., 2007). To evaluate T reg cell development in *Rag1*$^{-/-}$ bone marrow recipients, we examined recipient mice for the presence of Foxp3+ CD4+ thymocytes at early time points after bone marrow transfer. Analysis of recipient thymi 21 d after transplant (when the first wave of CD4+ thymocytes were first detected in the thymus) revealed a significant defect in T reg cell development (Fig. 9 E). The T reg cell

developmental defect was specific to *Rag1*$^{-/-}$ bone marrow recipients, as it was not observed in 6F/6F→B6 recipients, but was independent of the source of donor bone marrow cells (i.e., the percentage of Foxp3$^+$ CD4$^+$ thymocytes was significantly reduced in both 6Y/6Y→*Rag1*$^{-/-}$ and 6F/6F→*Rag1*$^{-/-}$ chimeras; Fig. 9 E). Although impaired, T reg cell development was not abrogated in *Rag1*$^{-/-}$ bone marrow recipients (Fig. 9 E). Collectively, these findings indicated that in 6F/6F→*Rag1*$^{-/-}$ bone marrow chimeras, the underlying defect in negative selection of 6F/6F thymocytes, combined with the partial block in T reg cell development and severe lymphopenia in *Rag1*$^{-/-}$ hosts renders these mice highly susceptible to autoimmune disease.

The 6Fζ mutation promotes TCR-mediated induction of Foxp3

We next sought to determine if the attenuated proximal TCR signaling in 6F/6F mice affects Foxp3 expression. Previous studies have shown that interruption of TCR signaling and/or selective antagonism of protein kinase B (Akt) results in the dephosphorylation and nuclear translocation of the transcription factors Foxo1 and Foxo3, which are required for the expression of Foxp3 (Merkenschlager and von Boehmer, 2010). Notably, thymocytes and T cells from 6F/6F mice exhibited a profound defect in Akt activation and Foxo1 phosphorylation after TCR engagement (Fig. 10, A and B; and not depicted).

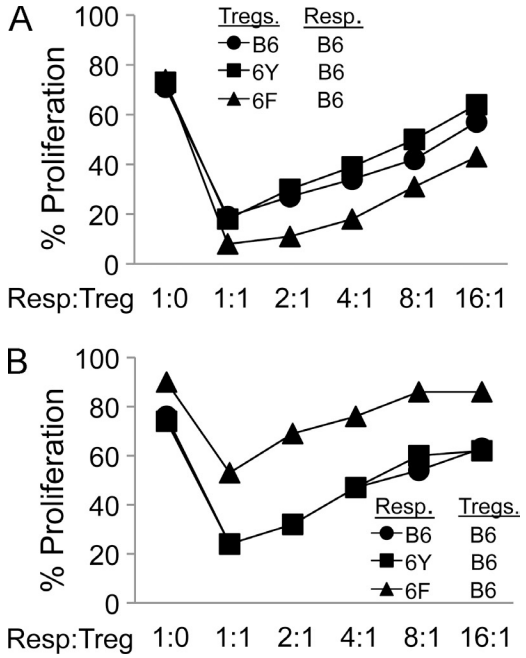

Figure 8. Analysis of 6F/6F T reg cells and T effector cells. (A) Suppression activity of CD25$^+$CD4$^+$ T cells (Tregs) from B6, 6Y/6Y, or 6F/6F mice against CD25$^-$CD4$^+$ responders (Resp) from B6 mice. Data shown are one representative of three independent experiments. (B) Suppression activity of CD25$^+$CD4$^+$ T cells (Tregs) from B6 mice against CD25$^-$CD4$^+$ responders (Resp) from B6, 6Y/6Y or 6F/6F mice. Data shown are one representative of three independent experiments.

In contrast, TCR-induced activation of NF-κB (Fig. 10 A) and TCR-induced calcium mobilization (Fig. 4 B), which are required for Foxp3 induction (Long et al., 2009; Oh-hora and Rao, 2009), appeared unaffected in 6F/6F cells. Moreover, a higher percentage of naive (CD44^{lo}CD25$^-$) CD4$^+$ T cells from 6F/6F mice expressed Foxp3 in response to either TCR stimulation followed by rest, or to constant TCR stimulation, a condition which was has been shown to be suboptimal for Foxp3 induction in wild-type T cells (Sauer et al., 2008; Fig. 10, C and D). The enhanced TCR-induced expression of Foxp3 by 6F/6F T cells was not caused by increased proliferation or survival of Foxp3$^+$ T cells relative to 6Y/6Y controls (unpublished data). Collectively, these results demonstrate that attenuation of proximal TCR signaling has a minimal impact on downstream pathways required for Foxp3 expression (NF-κB, and TCR-induced calcium mobilization) but markedly impairs TCR-induced activation of Akt which functions to inhibit Foxp3 expression.

DISCUSSION

In this study, we developed an experimental mouse model to evaluate the impact of TCR signal attenuation on T cell maturation and immune tolerance. We generated a knock-in allele under the control of the endogenous ζ regulatory elements that encodes a mutant (6F) ζ chain lacking functional ITAMs. Analysis of 6F/6F mice confirmed previous findings from several groups that reduction of TCR signaling potential impairs negative selection (Shores et al., 1997; Love et al., 2000; Pitcher et al., 2005a; Holst et al., 2008). Here, we identify quantitative defects in the activation of specific downstream signaling intermediates, including ERK5, Nur77, and Bim, as likely causes of the defect in negative selection. Although negative selection was defective and resulted in the survival of T cells that exhibited a phenotype consistent with high self-reactivity, 6F/6F mice did not develop autoimmune disease. A probable explanation for the maintenance of immune tolerance in 6F/6F mice is that T reg cell development is enhanced, resulting in a significant increase in the percentage and numbers of Foxp3$^+$ CD4$^+$ T cells in both the thymus and periphery. Although our current results do not provide unequivocal proof that the increase in T reg cells in 6F/6F mice compensates for the defect in negative selection and is required for the prevention of autoimmune disease, two findings strongly support this conclusion: first, we demonstrate that increased T reg cell/T effector ratios are needed to suppress proliferation of 6F/6F effector T cells in vitro, and second, development of 6F/6F T cells in 6F/6F:*Rag1*$^{-/-}$ bone marrow chimeric mice, where T reg cell maturation is impaired, results in severe, widespread autoimmune disease.

In addition to T reg cell numbers, T reg cell suppressive activity was significantly increased in 6F/6F mice. This could be caused by cell-intrinsic effects of TCR signal attenuation on T reg cell activity or, alternatively, to the fact that a higher percentage of conventional T cells in 6F/6F mice produce IL-2 in the absence of exogenous stimulation, as T reg cell survival and proliferation depend on exocrine-derived IL-2

A Autoimmune disease in Rag1$^{-/-}$ mice bone marrow recipients

Organ	6Y/6Y #1	6Y/6Y #2	6F/6F #1	6F/6F #2	6F/6F #3
lacrimal gland	-	-	++	++	+
salivary gland	+	-	++	++	+
liver	-	-	+	++	+
lung	-	+	++	++	++
kidney	-	-	+	++	++
intestine	-	-	-	++	+

Negative (-) : none or rare; + : 1 or 2 foci ; ++ : > 2 foci

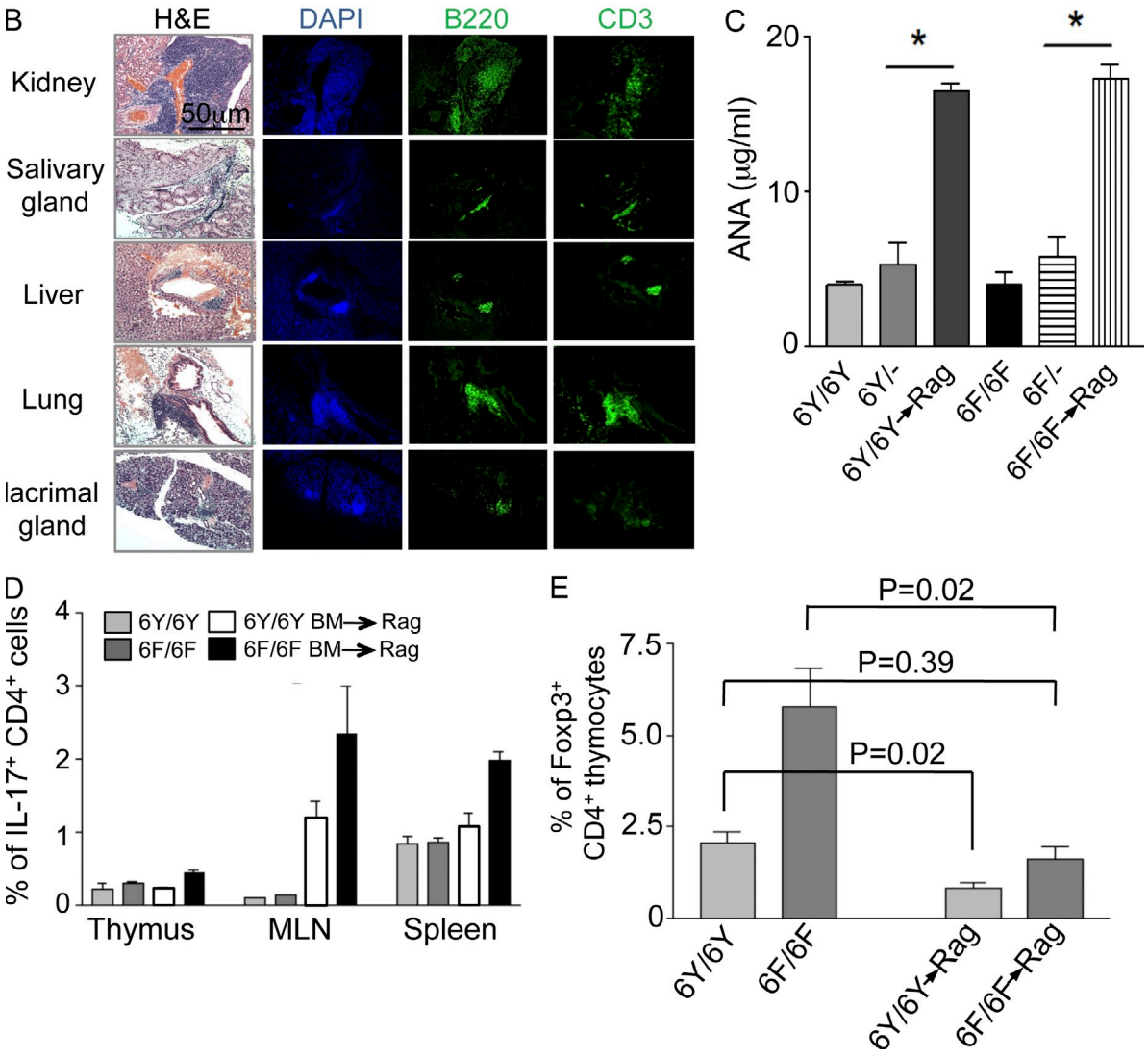

Figure 9. Transfer of 6F/6F bone marrow into *Rag1*$^{-/-}$ recipients results in autoimmune disease. (A) Incidence of inflammatory lesions in *Rag1*$^{-/-}$ bone marrow recipients 6 mo after transplantation of lineage-depleted 6Y/6Y and 6F/6F bone marrow cells. Hematoxylin and eosin (H&E)–stained sections of formalin fixed, paraffin-embedded tissues were examined for the presence of inflammatory lesions. Three sections obtained from different regions of the indicated tissues were examined. (B, left) Representative H&E-stained sections of tissues from *Rag1*$^{-/-}$ recipients of 6F/6F bone marrow 6 mo after transfer. (right) DAPI, and immunofluorescence (anti-B220 and anti-CD3) staining of sections adjacent to those shown in column 1. One representative experiment of three. (C) Serum anti-nuclear antibody (ANA) concentrations in 4–6-mo-old 6Y/6Y, 6Y/—, or 6F/F mice and in *Rag1*$^{-/-}$ recipients of 6Y/6Y or 6F/6F bone marrow 6 mo after adoptive bone marrow transfer. Sera were obtained from four mice from each group. (D) Percentage of IL-17$^+$ CD4$^+$ cells in 4–6-mo-old 6Y/6Y or 6F/F mice and in *Rag1*$^{-/-}$ recipients of 6Y/6Y or 6F/6F bone marrow 6 mo after adoptive bone marrow transfer. Four mice were analyzed for each group. (E) Percentage of Foxp3$^+$ CD4$^+$ thymocytes in 4-mo-old 6Y/6Y or 6F/6F mice or percentage of donor bone marrow–derived Foxp3$^+$ CD4$^+$ thymocytes 21 days after bone marrow transfer into irradiated *Rag1*$^{-/-}$ recipients. Three mice were analyzed for each group. For all bar graphs, data are mean ± SD. *, $P < 0.05$; **, $P < 0.01$.

produced by conventional (nonregulatory) T cells (Zheng and Rudensky, 2007). In any event, our results suggest that increased T reg cell development and function can compensate for an isolated (selective) defect in recessive tolerance (negative selection) caused by TCR signal attenuation.

We also provide an explanation for the discrepancy, with regard to susceptibility to autoimmune disease, between our results and those obtained using a model in which *Rag1*$^{-/-}$ mice were reconstituted with 6F retroviral infected bone marrow cells (Holst et al., 2008). T reg cell development is impaired in irradiated *Rag1*$^{-/-}$ recipient mice, especially at early time points after bone marrow transfer, and our results imply that this defect is at least partially responsible for the autoimmune disease in 6F/6F bone marrow recipient *Rag1*$^{-/-}$ mice. In this regard, it is notable that the increase in thymically derived T reg cells that we observed in 6F/6F mice was not seen in the analogous 6F/6F→ *Rag1*$^{-/-}$ model system, which resulted in severe autoimmune disease (Holst et al., 2008). The T reg cell developmental defects in that experimental model can most likely be attributed to thymus abnormalities in irradiated *Rag1*$^{-/-}$ recipients. *Rag1*$^{-/-}$ thymi exhibit both organizational and developmental defects that result from the absence of SP thymocytes, which are required for the expansion and maintenance of medullary epithelial cells (Klug et al., 2002; Shores et al., 1991). Adoptive transfer of bone marrow cells from wild-type mice into *Rag1*$^{-/-}$ mice leads to the eventual expansion and reorganization of the thymus, coincident with establishment of normal T cell development, into a structure with apparently normal cortical and medullary regions (Penit et al., 1996). However, our data suggest that reorganization of *Rag1*$^{-/-}$ thymi sufficient to support normal T reg cell development does not occur rapidly enough after bone marrow transfer to enforce tolerance on the first waves of developing donor thymocytes. Despite considerable peripheral expansion and/or peripheral generation of T reg cells in 6F/6F→*Rag1*$^{-/-}$ bone marrow chimeric mice, the defect in T reg cell development is apparently sufficient to trigger overt autoimmunity, perhaps similar to what is observed in T reg cell–depleted neonatal mice (Samy et al., 2008) or in *Rag*$^{-/-}$ mice that are injected with low numbers of T cells that contain a limited repertoire of T reg cells (Milner et al., 2007).

Negative selection and T reg cell development are thought to be directly controlled by the avidity and signal output of the TCR, with high avidity/strong signal inducing TCR-self interactions in the thymus favoring both outcomes (Jordan et al.,

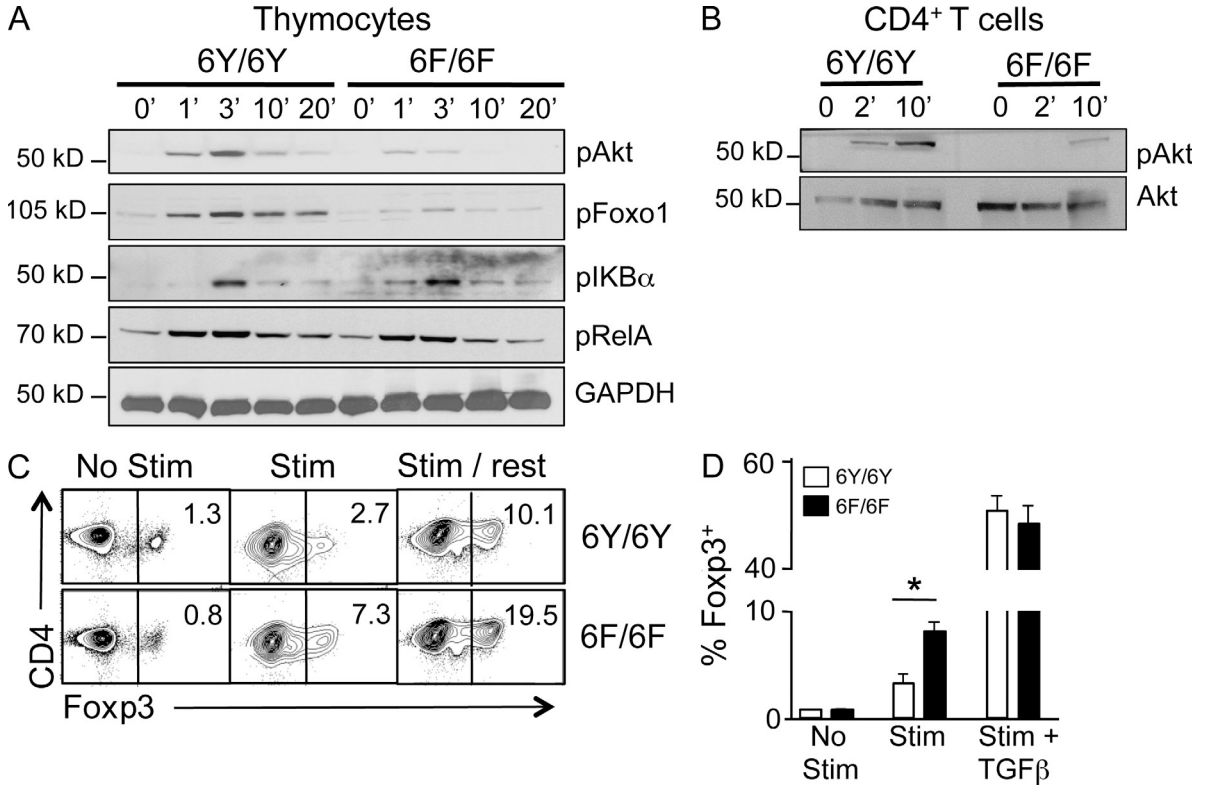

Figure 10. TCR-induced expression of Foxp3 is enhanced in 6F/6F T cells. Thymocytes (A) or lymph node CD4+ T cells (B) from 6Y/6Y and 6F/6F mice were stimulated with anti-CD3 and anti-CD4 for the indicated times, and whole-cell extracts were analyzed by SDS-PAGE and Western blotting with the indicated antibodies. One representative of two experiments. (C) Naive (CD44$^-$) CD25$^-$ CD4+ T cells were cultured for 44 h, without stimulation (No Stim), for 44 h with plate-bound anti-TCR + anti-CD28 (Stim), or for 18 h with anti-TCR + anti-CD28, and then replated without stimulation for 36 h without (Stim/Rest) or with TGFβ (Stim+TGFβ; not depicted). Cells were then counted, stained for CD4 and Foxp3, and analyzed by FACS. One representative of three experiments. (D) Summary of Foxp3 induction experiments described in C. Data are mean ± SD of three experiments. *, $P < 0.05$.

2001; Apostolou et al., 2002; Starr et al., 2003; Carter et al., 2005; Ordoñez-Rueda et al., 2009). Previous data have shown that increased TCR avidity or increased TCR signal intensity results in an increase in Foxp3$^+$ CD4$^+$ numbers in the thymus (Carter et al., 2005; Liston and Rudensky, 2007; Ordoñez-Rueda et al., 2009). However, this effect has been attributed to selective survival of T reg cells, which are relatively resistant to agonist-induced clonal deletion (negative selection) compared with conventional CD4$^+$ thymocytes, rather than to induced differentiation of T reg cells. Yet, resistance to clonal deletion cannot explain the increase in T reg cell numbers in 6F/6F mice, as TCR signaling potential is reduced and negative selection is impaired in these mice. Instead, our results support an instructive model for T reg cell development because they show that T reg cell production is quantitatively regulated by the number of positively selected thymocytes that express high self-affinity TCRs, particularly if these cells fail to receive signals sufficient to trigger negative selection. One explanation for our results is that TCR signal attenuation promotes the development or survival of precommitted T reg cell lineage cells in the thymus. Our findings are also consistent with, but do not specifically favor, a clonal diversion model for T reg cell maturation wherein uncommitted progenitor cells that express TCRs that bind with high-affinity to self-MHC/self-ligands are directed into the T reg cell lineage. According to this interpretation, T reg cell numbers are increased in 6F/6F mice because a larger percentage of thymocytes that express highly self-reactive TCRs fail to be negatively selected, and are therefore candidates for T reg cell lineage commitment.

Mutations that affect the activity of the scaffolding adapter protein LAT (LAT136YF; Sommers et al., 2005; Chuck et al., 2010) or the ZAP-70 tyrosine kinase (Sakaguchi et al., 2003; Siggs et al., 2007; Hsu et al., 2009; Tanaka et al., 2010), two signaling intermediates that function downstream of the TCR, lead to impaired negative selection and severe autoimmune disease. However, these mutations have been shown to inhibit the development of both conventional T cells and T reg cells (Koonpaew et al., 2006; Hsu et al., 2009; Au-Yeung et al., 2010; Tanaka et al., 2010), whereas our results demonstrate that T reg cell development and activity are not impaired, and in fact are enhanced, in 6F/6F mice. Thus, unlike 6F/6F mice, the T reg cell developmental and functional defects in LAY136YF and ZAP-70 mutant mice preclude the possibility that the defect in negative selection can be compensated for by a reciprocal increase in T reg cell development.

A striking and unexpected finding of our study was the differential effect of the 6F mutation on the activation of signaling pathways and intermediates downstream of the TCR. Although activation of TCR proximal effectors such as ZAP-70 was predictably reduced in 6F/6F thymocytes, the impact on more distal effectors was variable; for example, ERK, Cbl, and Akt phosphorylation were clearly reduced whereas calcium mobilization and activation of JNK, p38, and NF-κB were either less affected or unaffected. These results could reflect a specific requirement for ζ ITAMs for the activation of some but not all downstream pathways. Alternatively, consistent with the kinetic proofreading model for T cell activation (McKeithan, 1995), the differential sensitivity of specific pathways to TCR signal attenuation may reflect that there are different quantitative thresholds for the activation of individual downstream pathways, and that the activation kinetics of some responses are "analog" whereas others are "digital." Regardless of the mechanism, it is remarkable that pathways shown to be necessary for Foxp3 induction, such as calcium flux and NF-κB activation, were spared in 6F/6F cells, whereas those known to inhibit Foxp3 expression, such as Akt activation and Foxo1 phosphorylation, were strongly attenuated, suggesting that the TCR response to high-affinity ligands may be biased to favor the generation of T reg cells if proximal signals are attenuated.

In summary, the current results demonstrate that reduction of TCR signaling potential is not, by itself, sufficient to abrogate immune tolerance or result in autoimmune disease. Moreover, the finding that T reg cell development is potentiated under conditions where negative selection is severely impaired by reduction of TCR signaling potential reveals remarkable plasticity in the mechanisms that operate to enforce immune tolerance.

MATERIALS AND METHODS

Mice. The *Tcrζ* knock-in targeting vector was assembled using genomic fragments isolated from a BAC clone, RP24-354L20 (Children's Hospital Oakland Research Institute, Oakland, CA), a Frt-pgk-Neo-Frt-loxP cassette (gift from K. Pfeiffer, National Institute of Child Health and Human Development, Bethesda, MD) and a *diptheria toxin A chain* negative selection cassette (DTA; McCarrick et al., 1993). AND, H-Y, *Rag1*$^{-/-}$, B6, B6 (CD45.1), and B10.D2 (H-2^d) mice were obtained from Taconic Farms. Animal experiments were approved by the Animal Care and Use Committee of the National Institute of Child Health and Human Development, NIH.

Experimental autoimmune encephalitis (EAE). 6Y/6Y and 6F/6F mice used for EAE experiments were backcrossed to B6 for 8 generations. EAE induction was performed with a kit from Hooke Laboratories according to the manufacturer's instructions. Clinical scoring of EAE was as previously described (Reynolds et al., 2010).

Flow cytometry. Fluorescent dye–labeled antibodies to CD45.1 (A20), CD4 (RM4-5), CD5 (53.7.3), CD8 (53–6.7), CD24 (ML5), CD25 (PC61.5), CD44 (1M7), TCRβ (H57-597), CD69 (H1.2F3), CD62L (MEL-14), Annexin V, Vβ2(B20.6), Vβ3(KJ25), Vβ4 (KT4), Vβ5 (MR9-4), Vβ6 (RR4-7), Vβ8.1/2 (MR5-2), Vβ8.3 (1B3.3), Vβ9 (MR10-2), Vβ10b (B21.5), Vβ11(RR3-15), Vβ13 (MR12-3), Vβ14 (14–2), Vβ17a (KJ23), Vα2 (B20.1), Vα3.2 (RR3-16), Vα8.3 (B21.4), Vα11(RR8.1), TCRγδ (GL3), NK1.1 (PK136), CD49b (DX5), Ly6G/C (Gr-1), CD11b, Ter119, IL-17, IFN-γ, TNF, and IL-2 were purchased from BD. The lineage mix used for staining of DN thymocytes consisted of anti-CD4, -CD8, -TCRβ, -TCRγδ, -NK1.1, -DX5, -Gr-1, -CD11b, and -Ter 119. FoxP3 (FJK-16s), and Nur77 (12–5965) antibodies were purchased from eBioscience. Anti-FLAG (M2) was purchased from Sigma-Aldrich and anti-Myc (9B11) was obtained from Cell signaling. Cells were preincubated with blocking antibody (2.4G2) for 5 min, and then incubated for 15 min on ice with all specific antibodies. Samples were run on a FACSCalibur or LSRII cytometer (BD) and analyzed with FlowJo software (Tree Star). For intracellular cytokine staining, cells were incubated with brefeldin A for the final 2 h of a

5-h restimulation with PMA (phorbol 12-myristate 13-acetate) plus ionomycin. Permeabilization kits from eBioscience and BD were used for intracellular staining.

Cell stimulation and Western blotting. Thymocytes (10^7) or lymph node T cells (5×10^6) were stimulated at 37°C during the indicated periods of time with preformed immune complexes composed of biotinylated anti-CD3ε (10 mg/ml; 145-2C11; BD) and streptavidin (30 mg/ml; Sigma-Aldrich). When indicated, cells were stimulated with 100 mM pervanadate. After stimulation, cells were immediately resuspended in lysis buffer containing: 1% Triton X-100, 10 mM Tris, pH 7.4, 150 mM NaCl, 2 mM Na_3VO_4, 10 mM NaF, 1 mM EDTA, 1 protease inhibitor tablet (Roche). Cell equivalents (2×10^6 cells) were loaded on 4–12% gradient gels (Invitrogen) and analyzed by immunoblot. Antibodies used in this study included the following: antibody to Erk phosphorylated at Thr202 and Tyr204 (9101), antibody to Akt phosphorylated at Ser473 (9271), antibody to ZAP phosphorylated at Tyr319 (2701), antibody to JNK phosphorylated at Thr183/Tyr185 (9251), antibody to P38 phosphorylated at Thr180/Tyr182 (9211), antibody to ERK5 phosphorylated at Thr218/Tyr220 (3371) from Cell Signaling Technology; antibody to Vav phosphorylated at Tyr160 (44–482) from Invitrogen; antibody to SLP-76 phosphorylated at Tyr218 (558367) and to TCRβ chain (clone H57) from BD; antibody to CD3e (M-20) from Santa Cruz Biotechnologies. Anti-phosphotyrosine (4G10) was obtained from Millipore. Antibody to TCRζ chain was kindly provided by L. Samelson (National Cancer Institute, Bethesda, MD).

BrdU labeling experiments. For in vivo labeling of T cells, mice were supplied with drinking water containing BrdU (0.8mg/ml) for 3 d. BrdU staining was performed with a BrdU Flow kit from BD.

Cell purification. Purified T cell populations were obtained from lymph nodes using a magnetic bead/column system (MACS; Miltenyi Biotec). Purity for isolated $CD4^+$ and $CD8^+$ T Cells was >95%. For the T reg cell suppression assays, $CD25^-$ (responders) and $CD25^+$ (T reg cells) $CD4^+$ T cells were isolated by first labeling total lymph node cells with anti-B220 biotin, anti-CD11b biotin, anti-CD8 biotin, and anti-CD25 PE. $CD4^+$ cells (unlabeled fraction) were isolated by magnetic columns after being washed and labeled with streptavidin microbeads. Purified $CD4^+$ cells were labeled with anti–CD25-PE microbeads, washed, and run through another magnetic column. $CD25^-$ cells were obtained from the flow-through of the column, and $CD25^+$ cells were obtained by elution of bound cells. Purity for both $CD25^-$ and $CD25^+$ $CD4^+$ subsets was >90%.

CFSE cell labeling. Cells were labeled with 5 μg/ml of CFSE (Invitrogen) for 15 min at 37°C. Cell division was assessed by determining the percentage of $CFSE^{lo/med}$ cells by FACS and dividing by the percentage of $CFSE^{lo/med}$ cells obtained after stimulation with PMA and ionomycin.

T reg cell suppression assay. Magnetic bead purified $CD25^-$ $CD4^+$ and $CD25^+$ $CD4^+$ lymph node T cells were used as responder T cells and T reg cells, respectively. CFSE-labeled responder T cells (5×10^4) were cultured for 72 h with irradiated splenocytes (5×10^4) and anti-CD3 (1 μg/ml, 2C11, BioXCell) in the presence or absence of the indicated ratio of $CD25^+$ $CD4^+$ cells. The division of responder T cells was assessed by determining the dilution of CFSE by FACS. Proliferation was shown as a percentage of maximum in the absence of T reg cells.

In vitro Foxp3 induction assay. The in vitro Foxp3 induction assay was performed as previously described (Sauer et al., 2008).

Generation of bone marrow chimeras. Donor bone marrow cells were depleted of lineage-positive cells by the addition of anti-CD3, anti-CD4, anti-CD8, anti-CD11b, and anti-CD19 antibodies, followed by purification using the magnetic column system from Miltenyi Biotec. 5×10^6 cells were injected into the tail veins or retro-orbital sinus of irradiated B6 (650 rads) or *Rag1*$^{-/-}$ mice.

Histopathology and immunohistochemistry. Mouse tissues were fixed in 4% paraformaldehyde (Protocol) and embedded in paraffin. 6-μm sections were stained with hematoxylin and eosin, or, for immunofluorescence staining, tissue sections were fixed in 4% (wt/vol) paraformaldehyde and blocked with 1% (wt/vol) BSA and 10% (vol/vol) goat serum in PBS before incubation with FITC-conjugated rat anti–mouse B220 (abCAM) or rat anti–mouse CD3 (abCAM). Images were obtained on an Axiovert LSM 510-META inverted microscope (Carl Zeiss), except for tiled images, which were captured and assembled on a Fluoview FV1000 (Olympus).

Real-time PCR. For gene-expression studies, total cell RNA was isolated with a PicoPure RNA Isolation kit (Arcturus). RNA samples (100 ng of each) were reverse-transcribed with the SuperScript First-Strand Synthesis system (Invitrogen) and were assayed by RT-PCR. Transcripts were quantified with a Roche LightCycler 480. Duplicates were run for each sample in a 96-well plate; *Actb* (encoding b-actin) served as the endogenous reference gene. The relative quantification method was used, with the ratio of the mRNA abundance of the gene of interest normalized to the abundance of β-actin mRNA and with the average of control thymocyte samples serving as the calibrator value. The specificity of the products was confirmed by melting curves and electrophoresis.

Statistical analysis. Statistical analysis was performed with two-tailed, unpaired Student's *t* tests with Prism soft ware. For all figures: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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