

Roles of the Src Tyrosine Kinases Lck and Fyn in Regulating $\gamma\delta$ TCR Signal Strength

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Abstract

Lck and Fyn, members of the Src family of tyrosine kinases, are key components of the $\alpha\beta$ TCR-coupled signaling pathway. While it is generally accepted that both Lck and Fyn positively regulate signal transduction by the $\alpha\beta$ TCR, recent studies have shown that Lck and Fyn have distinct functions in this signaling pathway, with Lck being a positive regulator and Fyn being a negative regulator of $\alpha\beta$ TCR signal transduction. To determine whether Lck and Fyn also differentially regulate $\gamma\delta$ TCR signal transduction, we analyzed $\gamma\delta$ T cell development and function in mice with reduced Lck or Fyn expression levels. We found that reducing Lck or Fyn levels altered the strength of the $\gamma\delta$ TCR signaling response, with low levels of Lck weakening $\gamma\delta$ TCR signal strength and low levels of Fyn augmenting $\gamma\delta$ TCR signal strength. These alterations in $\gamma\delta$ TCR signal strength had profound effects not only on $\alpha\beta/\gamma\delta$ lineage choice, but also on $\gamma\delta$ thymocyte maturation and $\gamma\delta$ T cell effector function. These results indicate that the cellular levels of Lck and Fyn play a role in regulating the strength of the $\gamma\delta$ TCR signaling response at different stages in the life of the $\gamma\delta$ T cell.

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Introduction

Signaling by the TCR is required at multiple stages in the life of a T cell. In the thymus, TCR signaling is necessary for lineage commitment and repertoire selection, while in the periphery, TCR signaling is necessary for maintenance of the peripheral T cell pool and for activation and differentiation of mature T cells. Lck and Fyn, two members of the Src family of tyrosine kinases (SFKs), are involved in initiating the TCR-coupled signaling cascade [1,2]. Following TCR engagement, Lck and/or Fyn phosphorylate the tyrosines within the ITAMs of the CD3 and TCR ζ chains. This proximal signaling event leads to the recruitment of other signaling molecules to the TCR signaling complex and to the subsequent activation of signaling pathways that ultimately lead to the nucleus and initiation of gene transcription.

It is generally accepted that both Lck and Fyn positively regulate signal transduction by the $\alpha\beta$ TCR because, in the absence of either one of these SFKs, $\alpha\beta$ TCR signaling responses are impaired following anti-CD3 mAb stimulation [3–9]. However, it has also been shown that Lck and Fyn localize to different subcellular compartments [10,11] and have different substrates [11,12], suggesting that they have discrete functions during $\alpha\beta$ T cell activation. This idea is supported by the disparate phenotypes of Lck- and Fyn-deficient mice. In Lck $^{-/-}$ mice, thymus cellularity is severely reduced, thymocyte development is almost completely blocked at the CD4 $^+$ CD8 $^+$ (double-positive; DP) stage, and very few mature $\alpha\beta$ TCR $^+$ cells are detected in peripheral lymphoid tissues [12–14]. In contrast, Fyn $^{-/-}$ mice exhibit a mild defect in $\alpha\beta$ T cell development, as shown by the fact that Fyn $^{-/-}$

thymocytes, when *in vitro* stimulated, do not flux calcium or proliferate as well as wild-type (WT) thymocytes [3,5]. Despite this signaling defect in the thymus, equivalent numbers of $\alpha\beta$ T cells are found in the periphery of Fyn $^{-/-}$ and WT mice [3,5].

While recent studies have confirmed that Lck functions primarily as a positive regulator of $\alpha\beta$ TCR signaling [15–17], evidence is accumulating in support of Fyn acting as a negative regulator of $\alpha\beta$ TCR signaling. First, it has been shown that Fyn is responsible for phosphorylating the adaptor protein, phosphoprotein associated with glycolipid-enriched membranes or PAG, in both resting thymocytes and T cells [11]. Once phosphorylated, PAG then recruits Csk, an inhibitor of SFKs [18]. Recruitment of Csk to phosphorylated PAG is required for optimal Csk kinase activity because, in the absence of Fyn, there is reduced phosphorylation of PAG and reduced Csk kinase activity [11]. Therefore, by indirectly controlling the activity of the inhibitor Csk, Fyn may negatively regulate the activation threshold of $\alpha\beta$ T cells [11]. It has also been shown that CD8 $^+$ T cells from Fyn $^{-/-}$ F5 $\alpha\beta$ TCR Tg mice are hyperresponsive in comparison to CD8 $^+$ T cells from WT F5 $\alpha\beta$ TCR Tg mice following *in vitro* stimulation with peptide and APCs [19]. This hyperresponsiveness is manifested as enhanced proliferation, increased IL-2 production and more effective cytolytic activity [19]. CD4 $^+$ T cells from Fyn $^{-/-}$ DO11.10 $\alpha\beta$ TCR Tg mice, however, do not display increased proliferation compared to CD4 $^+$ T cells from WT DO11.10 $\alpha\beta$ TCR Tg mice when stimulated, either *in vitro* or *in vivo*, with peptide and APCs [20]. Nonetheless, when activated under the appropriate priming conditions, CD4 $^+$ T cells from Fyn $^{-/-}$ DO11.10 $\alpha\beta$ TCR Tg mice produce significantly more IL-4 or IFN γ than CD4 $^+$ T cells from WT DO11.10 $\alpha\beta$ TCR Tg mice

[20]. Taken together, these findings suggest that Fyn negatively regulates the $\alpha\beta$ TCR signaling response.

Since studies investigating the functions of Lck and Fyn have focused primarily on $\alpha\beta$ T cells, it is not known whether their functional dichotomy is observed in only $\alpha\beta$ T lineage cells or in both $\alpha\beta$ and $\gamma\delta$ T lineage cells. Analyses of Lck^{-/-} and Lck^{-/-}Fyn^{-/-} mice have in fact revealed differences in the requirements for these SFKs in $\alpha\beta$ and $\gamma\delta$ T cell development. In Lck^{-/-} mice, the number of thymic and peripheral $\gamma\delta$ T cells is only modestly reduced compared to their numbers in WT mice [13,14,21]. Moreover, in Lck^{-/-}Fyn^{-/-} mice, in which $\alpha\beta$ T cell development is completely abrogated, a small number of $\gamma\delta$ TCR⁺ cells do develop and can be detected in secondary lymphoid tissues, the small intestine, and the epidermis [21,22]. These differential requirements for Lck and Fyn in $\alpha\beta$ and $\gamma\delta$ T cell development suggest that these SFKs may have different functions in $\alpha\beta$ - and $\gamma\delta$ TCR signal transduction. To investigate this, we evaluated the individual roles of Lck and Fyn in the development and function of $\gamma\delta$ lineage cells. Here, we report that Lck and Fyn expression levels vary in $\gamma\delta$ lineage cells depending on their stage in development, with thymic $\gamma\delta$ T cells expressing relatively high levels of Lck and Fyn and peripheral $\gamma\delta$ T cells expressing relatively low levels of Lck and Fyn. These differences in the cellular levels of Lck and Fyn play a role in regulating the strength of the $\gamma\delta$ TCR signaling response at the different developmental stages because, when we reduced Lck or Fyn expression levels by using Lck^{+/-} and Fyn^{+/-} mice, we observed significant effects on $\alpha\beta/\gamma\delta$ lineage choice, $\gamma\delta$ thymocyte maturation, and $\gamma\delta$ T cell effector function. Moreover, because reducing the levels of Lck or Fyn altered the $\gamma\delta$ TCR signaling response, such that low Lck levels weakened $\gamma\delta$ TCR signal strength and low Fyn levels augmented $\gamma\delta$ TCR signal strength, we conclude that Lck and Fyn have similar functions in $\alpha\beta$ - and $\gamma\delta$ TCR signal transduction, with Lck serving to amplify the TCR signal and Fyn serving to dampen the TCR signal.

Results

Expression Pattern of Lck and Fyn in $\gamma\delta$ Lineage Cells

Although it is generally accepted that $\gamma\delta$ T cells express Lck and Fyn, this idea is based more on indirect evidence from studies investigating $\gamma\delta$ T cell development in Lck- or Fyn-deficient mice [13,14,21,23,24] than on a direct demonstration of expression [25]. To resolve this, we developed an intracellular (i.c.) flow cytometric assay to measure and compare the relative levels of Lck and Fyn in $\alpha\beta$ and $\gamma\delta$ lineage cells from wild-type (WT) mice. Using Lck^{-/-} and Fyn^{-/-} cells as negative staining controls, we found that both Lck and Fyn are expressed in DN $\gamma\delta$ thymocytes and peripheral DN $\gamma\delta$ T cells (Fig. 1A). On average, DN $\gamma\delta$ thymocytes expressed Lck and Fyn at higher levels than DP and mature CD4⁺ thymocytes, whereas peripheral DN $\gamma\delta$ T cells expressed Lck at levels comparable to those in CD4⁺ T cells and Fyn at levels lower than those in CD4⁺ T cells (Fig. 1A). When Lck and Fyn expression levels were compared between thymic and peripheral $\gamma\delta$ T cells, we found that both SFKs are expressed at significantly higher levels in DN $\gamma\delta$ thymocytes than in DN $\gamma\delta$ T cells (Fig. 1B–C). This finding suggested that the expression levels of both Lck and Fyn are down-regulated once $\gamma\delta$ T cells emigrate from the thymus to the secondary lymphoid organs. This same phenomenon was also observed for $\alpha\beta$ lineage cells; however, the degree of reduction in Lck and Fyn expression levels between the thymus and LN was greater for $\gamma\delta$ lineage cells than $\alpha\beta$ lineage cells (Fig. 1C and data not shown). Taken together, these data indicated that immature and mature $\gamma\delta$ lineage cells express Lck

and Fyn and that the expression of these SFKs is dynamic during $\gamma\delta$ T cell development and maturation.

Polyclonal $\gamma\delta$ T Cell Development in Lck^{+/-} and Fyn^{+/-} Mice

Because DN $\gamma\delta$ thymocytes expressed higher levels of Lck and Fyn than mature DN $\gamma\delta$ T cells, we sought to determine whether high levels of Lck or Fyn were required for $\gamma\delta$ lineage commitment and/or development in the thymus. To investigate this, we reduced the expression levels of Lck or Fyn during T cell development by using Lck^{+/-} and Fyn^{+/-} mice. To verify that protein expression was reduced in the heterozygous mice, we compared the relative expression levels of Lck and Fyn in immature and mature CD4⁺ lineage cells from Lck^{+/-} and Fyn^{+/-} mice with those from WT mice. As expected, we observed a 50% reduction in Lck expression levels in Lck^{+/-} mice and a 50% reduction in Fyn expression levels in Fyn^{+/-} mice (Fig. 2A). In addition, there was no compensatory increase in the expression of one SFK when expression of the other SFK was reduced (Fig. 2A).

When $\gamma\delta$ T cell development was analyzed in Lck^{+/-} and Fyn^{+/-} mice, we observed no significant difference in the number of DN $\gamma\delta$ TCR⁺ cells in the thymus and lymph nodes (LNs) of these mice compared to WT mice (Fig. 2B). Moreover, phenotypic analysis of the DN $\gamma\delta$ thymocytes and mature DN $\gamma\delta$ T cells from WT, Lck^{+/-} and Fyn^{+/-} mice revealed no appreciable differences in V γ usage, TCR $\gamma\delta$ surface levels and cell surface phenotype (data not shown), indicating that reducing the expression levels of Lck or Fyn resulted in no apparent defect in the development of polyclonal $\gamma\delta$ T cells.

Because $\gamma\delta$ T cell development appeared not to be affected in Lck^{+/-} and Fyn^{+/-} mice, we examined the levels of Lck and Fyn in $\gamma\delta$ lineage cells of the heterozygous mice to determine whether they were reduced by 50% as they were in $\alpha\beta$ lineage cells (Fig. 2A and C). We found that Lck levels were indeed reduced by 50% in thymic and peripheral $\gamma\delta$ lineage cells from Lck^{+/-} mice (Fig. 2C). Fyn levels, on the other hand, were reduced to ~60% in immature and mature $\gamma\delta$ lineage cells from Fyn^{+/-} mice, which is significantly different from the expected 50% (Fig. 2C). Since Fyn expression levels in $\gamma\delta$ lineage cells were not reduced to the expected 50% in Fyn^{+/-} mice, we determined whether the thymic precursors in Lck^{+/-} and Fyn^{+/-} mice displayed a 50% reduction in Lck and Fyn expression levels, respectively. To accomplish this, we compared Lck and Fyn expression levels in lineage-negative CD44⁺CD25⁺ (DN2) thymocytes from WT, Lck^{+/-} and Fyn^{+/-} mice, as this thymocyte subset contains precursors that have the potential to develop into $\alpha\beta$ or $\gamma\delta$ lineage cells [26,27]. We found that, while Lck expression levels were reduced to 50% in Lck^{+/-} DN2 thymocytes, Fyn expression levels were only reduced to 85% in Fyn^{+/-} DN2 thymocytes (Fig. 2D and E). These data demonstrated that although Fyn levels are reduced in thymic precursors and $\gamma\delta$ lineage cells from Fyn^{+/-} mice, they are not reduced by 50% as they are in $\alpha\beta$ lineage cells.

Effect of Reducing Lck or Fyn Levels on the Commitment and Development of $\gamma\delta$ Lineage Cells

Another reason why we may not have observed any defects in $\gamma\delta$ T cell development in Lck^{+/-} and Fyn^{+/-} mice is because thymocyte development and selection are able to compensate for alterations in $\gamma\delta$ TCR signal transduction, which may result from reductions in Lck or Fyn expression. To address this, we mated a $\gamma\delta$ TCR transgene onto Lck^{+/-} and Fyn^{+/-} genetic backgrounds to determine whether fixing the specificity of the $\gamma\delta$ TCR revealed defects in $\alpha\beta/\gamma\delta$ lineage choice and/or $\gamma\delta$ T cell development. For

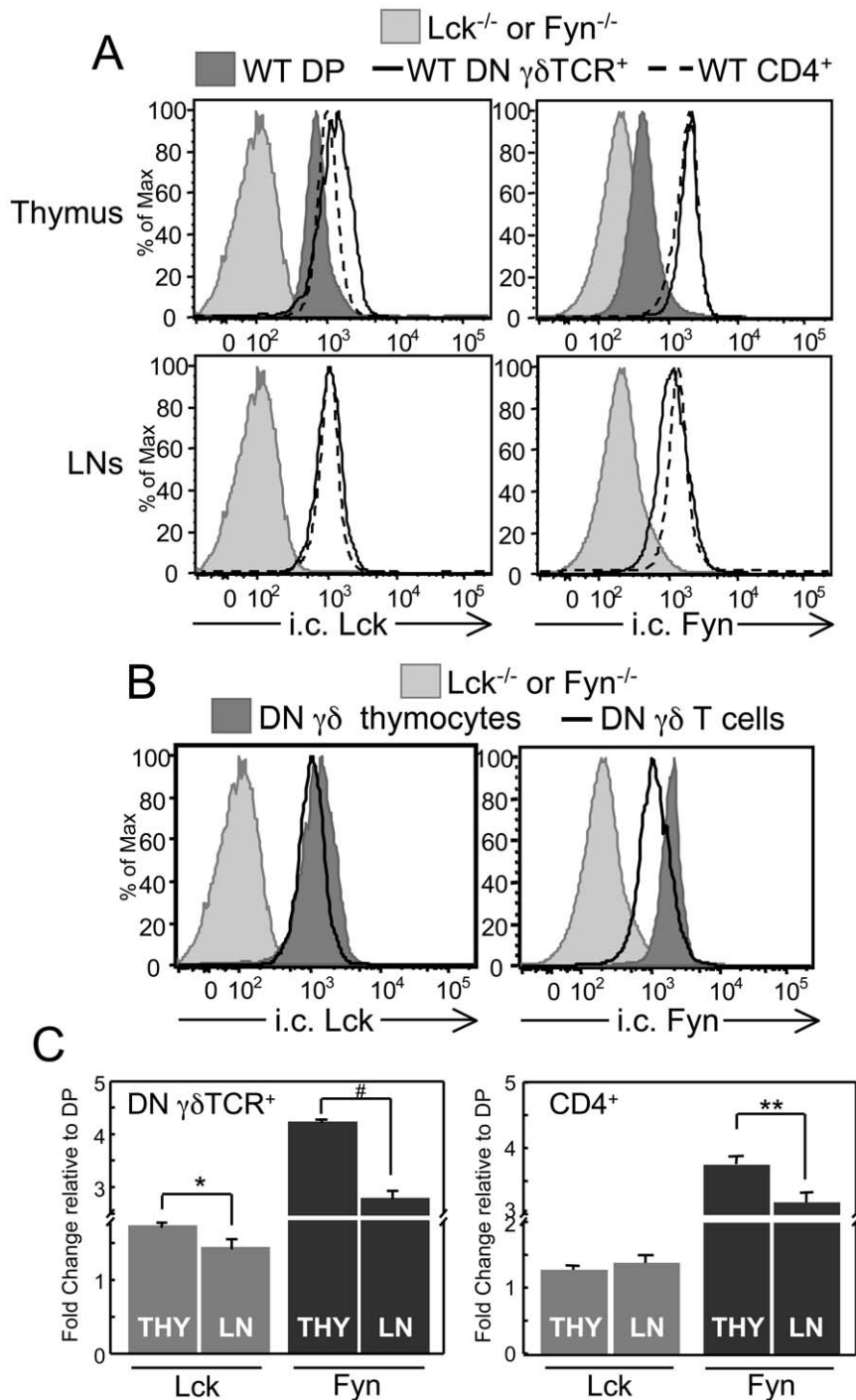


Figure 1. Flow cytometric analysis of the intracellular levels of Lck and Fyn in $\gamma\delta$ lineage cells. A. Histograms show representative staining of the i.c. levels of Lck and Fyn in gated populations of DP thymocytes and of thymic and LN CD4⁺ CD3⁺ and DN $\gamma\delta$ TCR⁺ cells from WT (B6) mice. Staining of cells from Lck^{-/-} and Fyn^{-/-} mice are shown as negative controls for i.c. staining of Lck and Fyn, respectively. B. Comparison of the relative expression levels of Lck and Fyn in gated DN $\gamma\delta$ TCR⁺ thymocytes and LN cells and, for comparison, CD4⁺ CD3⁺ thymocytes and LN cells. Lck and Fyn expression levels in immature and mature subsets were normalized to those of DP thymocytes, as this population had, in every experiment, consistently lower levels of Lck and Fyn than any other thymocyte or T cell subset (see A). Data are presented as fold change relative to DP thymocytes (set to 1). Data are representative of at least 6 independent experiments. Bars represent mean \pm SEM. * $p \leq 0.05$, ** $p \leq 0.01$, # $p \leq 0.001$. doi:10.1371/journal.pone.0008899.g001

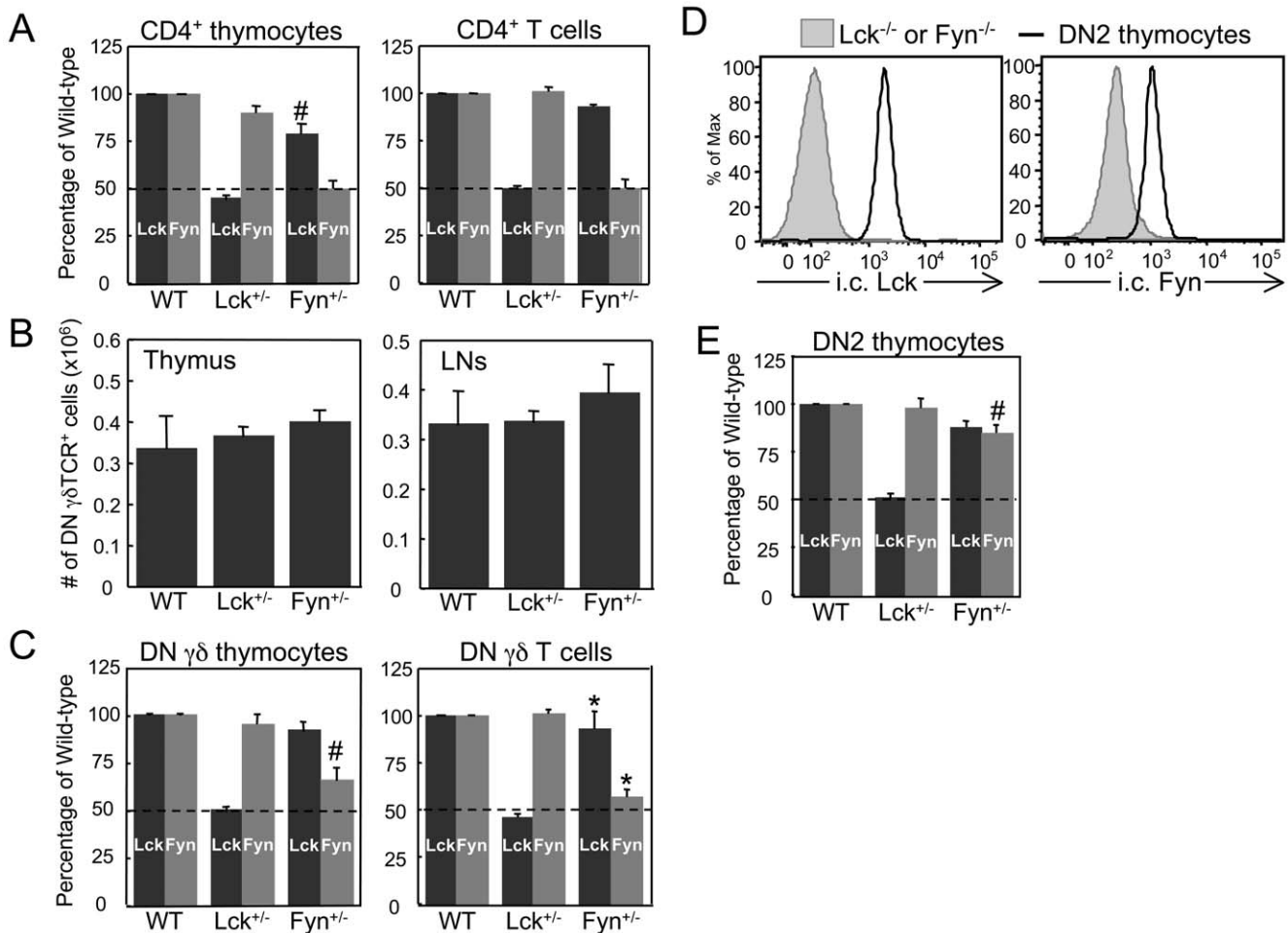


Figure 2. Effect of reducing Lck or Fyn levels on polyclonal $\gamma\delta$ T cell development. A. Demonstration of the reduction of Lck or Fyn levels in CD4⁺ thymocytes and LN cells from Lck^{+/-} and Fyn^{+/-} mice. The MFI of the i.c. levels of Lck and Fyn in CD4⁺ lineage cells from heterozygous mice are expressed as a percentage of the MFI of the i.c. levels of Lck and Fyn in CD4⁺ lineage cells from WT mice. A dashed line marks the expected 50% reduction in WT Lck and Fyn levels. B. Number of DN $\gamma\delta$ thymocytes and LN $\gamma\delta$ T cells in WT, Lck^{+/-}, and Fyn^{+/-} mice. Data represent at least 6 mice per genotype. C. Quantifying the reduction of Lck and Fyn expression levels in DN $\gamma\delta$ TCR⁺ thymocytes and LN cells from Lck^{+/-} and Fyn^{+/-} mice. The MFI of the i.c. levels of Lck and Fyn in $\gamma\delta$ lineage cells from heterozygous mice are expressed as a percentage of the MFI of the i.c. levels of Lck and Fyn in $\gamma\delta$ lineage cells from WT mice. A dashed line marks the expected 50% reduction in WT Lck and Fyn levels. D. Relative expression levels of Lck and Fyn in WT DN2 (lin⁻ CD25⁺ CD44⁺) thymocytes, where lin⁻ is defined as CD4⁻ CD8⁻ CD11b⁻ TCR β ⁻ TCR γ ⁻ CD19⁻ NK1.1⁻ IA^P⁻ Ly6-G/Ly6-C⁻. E. Quantifying the reduction of Lck and Fyn expression levels in DN2 thymocytes from Lck^{+/-} and Fyn^{+/-} mice. The MFI of the i.c. levels of Lck and Fyn in DN2 thymocytes from heterozygous mice are expressed as a percentage of the MFI of the i.c. levels of Lck and Fyn in DN2 thymocytes from WT mice. A dashed line marks the expected 50% reduction in WT Lck and Fyn levels. In A, B, C, and E, the bars represent mean \pm SEM. * $p \leq 0.05$, ** $p \leq 0.01$, # $p \leq 0.001$.

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these experiments, we used the V γ 6/J γ 1/C γ 1 and V δ 1/D δ 1/J δ 2/C δ transgenic ($\gamma\delta$ TCR Tg) mouse [28], which we have previously used to study $\alpha\beta/\gamma\delta$ lineage choice, $\gamma\delta$ T cell development and $\gamma\delta$ TCR signal transduction [29–31]. It is important to note that, although V γ 6/V δ 1⁺ $\gamma\delta$ T cells are only generated in the fetal thymus of a WT (non- $\gamma\delta$ TCR Tg) mouse [28], the V γ 6/V δ 1⁺ $\gamma\delta$ T cells generated in the $\gamma\delta$ TCR Tg mouse represent adult $\gamma\delta$ T cells, as they express the panel of $\gamma\delta$ -biased genes [31] typical of adult but not fetal $\gamma\delta$ T cell populations [32]. Moreover, one of the advantages of using this $\gamma\delta$ TCR Tg mouse model to study $\alpha\beta/\gamma\delta$ lineage choice is that the rearranged TCR γ and δ chains are expressed early during T cell development prior to TCR β expression and, as a consequence, the $\alpha\beta/\gamma\delta$ lineage decision is mediated exclusively by the $\gamma\delta$ TCR [29].

By fixing the specificity of the $\gamma\delta$ TCR, we observed significant effects on the $\alpha\beta/\gamma\delta$ lineage fate decision when the levels of Lck

but not Fyn were reduced. In WT $\gamma\delta$ TCR Tg mice, equivalent numbers of DN $\gamma\delta$ TCR⁺ thymocytes ($\gamma\delta$ lineage) and DP thymocytes ($\alpha\beta$ lineage cells) are generated (Fig. 3A–B). Reducing Lck expression resulted in a striking 4-fold increase in thymus cell number compared to WT $\gamma\delta$ TCR Tg mice, most likely due to the significant increase in the percentage of DP thymocytes (Fig. 3A). Consequently, the number of $\alpha\beta$ lineage cells in Lck^{+/-} $\gamma\delta$ TCR Tg mice was significantly higher than that in WT $\gamma\delta$ TCR Tg mice (Fig. 3B). These findings are consistent with those of a previous study, in which $\gamma\delta$ TCR-dependent generation of DP thymocytes but not of DN $\gamma\delta$ TCR⁺ thymocytes was observed in the absence of Lck expression [33]. In contrast, reducing Fyn levels had no effect on total thymus cell number. Nonetheless, we did observe a decrease in both the percentage and number of DP thymocytes in Fyn^{+/-} $\gamma\delta$ TCR Tg mice, although these differences were not statistically significant from the percentage and number of DP

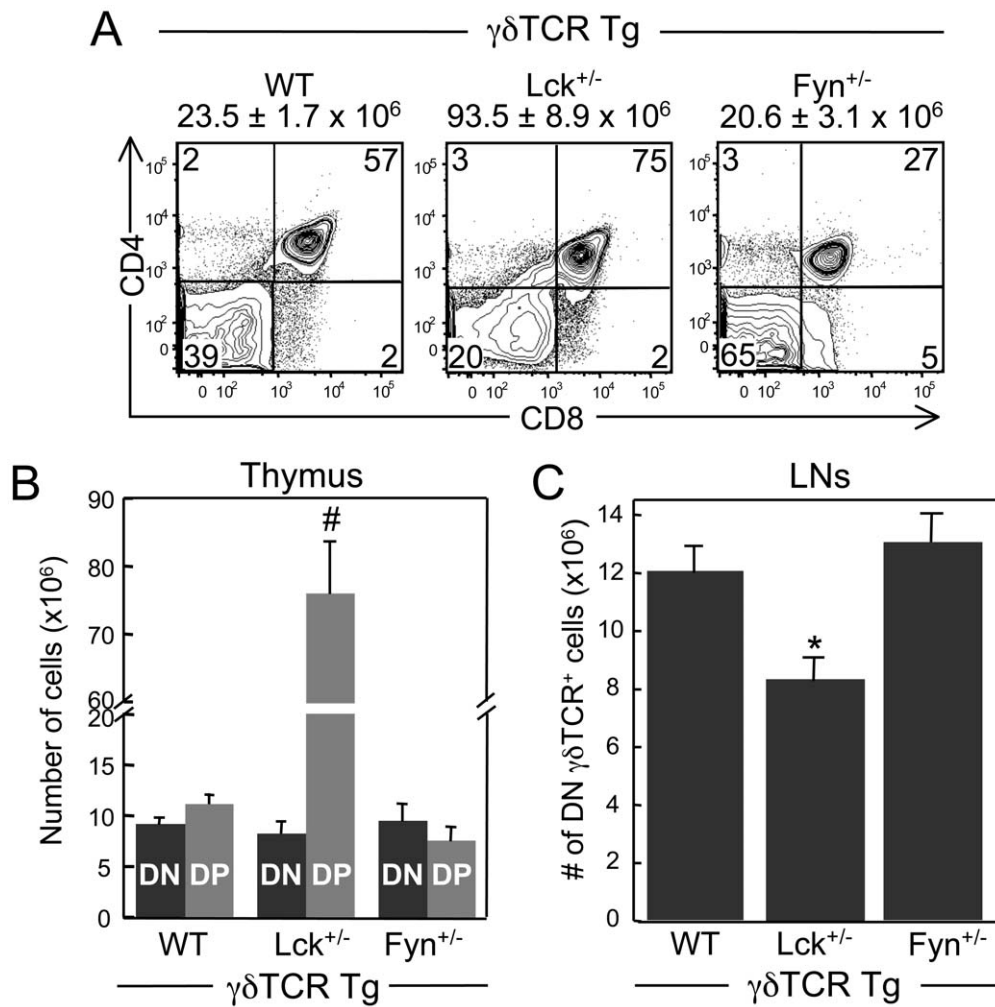


Figure 3. Effect of reducing Lck or Fyn levels on $\alpha\beta/\gamma\delta$ lineage commitment and $\gamma\delta$ T cell development. A. Dot plots show representative CD4 versus CD8 staining profiles for WT $\gamma\delta$ TCR Tg, Lck^{+/-} $\gamma\delta$ TCR Tg, and Fyn^{+/-} $\gamma\delta$ TCR Tg thymocytes. Numbers in the quadrants represent percentage of cells in each quadrant. The mean thymus cell number \pm SEM for each genotype are displayed above the respective two-color plot. B. Mean number of DN (DN $\gamma\delta$ TCR⁺; $\gamma\delta$ lineage) and DP ($\alpha\beta$ lineage) thymocytes in WT $\gamma\delta$ TCR Tg, Lck^{+/-} $\gamma\delta$ TCR Tg and Fyn^{+/-} $\gamma\delta$ TCR Tg mice. Data represent at least 6 mice per genotype. C. Mean number of DN $\gamma\delta$ T cells in the LNs of WT $\gamma\delta$ TCR Tg, Lck^{+/-} $\gamma\delta$ TCR Tg and Fyn^{+/-} $\gamma\delta$ TCR Tg mice. Data represent at least 5 mice per genotype. In B and C, the bars represent mean \pm SEM. * $p \leq 0.05$, # $p \leq 0.001$. doi:10.1371/journal.pone.0008899.g003

thymocytes in WT $\gamma\delta$ TCR Tg mice (Fig. 3A–B). Interestingly, despite the changes in the numbers of DP thymocytes in the heterozygous mice, the numbers of DN $\gamma\delta$ TCR⁺ thymocytes, or $\gamma\delta$ lineage cells, in Lck^{+/-} $\gamma\delta$ TCR Tg and Fyn^{+/-} $\gamma\delta$ TCR Tg mice were comparable to their number in WT $\gamma\delta$ TCR Tg mice (Fig. 3A–B).

It has previously been shown that genetic manipulation of $\gamma\delta$ TCR signal strength affects $\alpha\beta/\gamma\delta$ lineage choice in a consistent manner. Namely, when the $\gamma\delta$ TCR signaling response is strengthened, $\gamma\delta$ lineage fate is favored and, conversely, when the $\gamma\delta$ TCR signaling response is weakened, $\alpha\beta$ lineage fate is favored [29,33]. Our finding that the number of $\alpha\beta$ lineage cells was significantly increased in Lck^{+/-} $\gamma\delta$ TCR Tg mice compared to WT $\gamma\delta$ TCR Tg mice suggested that reducing Lck levels weakened $\gamma\delta$ TCR signal strength. Surprisingly, this increase in the number of DP thymocytes was not accompanied by a corresponding decrease in the number of DN $\gamma\delta$ thymocytes in Lck^{+/-} $\gamma\delta$ TCR Tg (Fig. 3B). We reasoned that the number of DN $\gamma\delta$ thymocytes in Lck^{+/-} $\gamma\delta$ TCR Tg mice may not reflect the number of thymocytes that adopted the $\gamma\delta$ fate but instead

reflected an expansion of the thymocytes that already adopted the $\gamma\delta$ fate. To investigate this, we compared the proliferative status of DN $\gamma\delta$ thymocytes in WT $\gamma\delta$ TCR Tg, Lck^{+/-} $\gamma\delta$ TCR Tg, and Fyn^{+/-} $\gamma\delta$ TCR Tg mice by measuring their expression of the Ki-67 Ag, which is a marker of actively cycling cells [34,35]. We found that the frequency of Ki67⁺ DN $\gamma\delta$ thymocytes in Lck^{+/-} $\gamma\delta$ TCR Tg mice was significantly higher than the frequency of Ki67⁺ DN $\gamma\delta$ thymocytes in WT $\gamma\delta$ TCR Tg and Fyn^{+/-} $\gamma\delta$ TCR Tg mice (Table 1). These data suggested that the number of DN $\gamma\delta$ thymocytes in Lck^{+/-} $\gamma\delta$ TCR Tg mice does not reflect the number of thymocytes that adopted the $\gamma\delta$ fate.

Next, we compared the expression levels of the $\gamma\delta$ TCR and CD5 on the surface of DN $\gamma\delta$ thymocytes from WT $\gamma\delta$ TCR Tg, Lck^{+/-} $\gamma\delta$ TCR Tg, and Fyn^{+/-} $\gamma\delta$ TCR Tg mice to gauge the effects of reducing Lck or Fyn levels on the phenotype of the cells choosing the $\gamma\delta$ lineage. When we examined $\gamma\delta$ TCR and CD5 surface levels on the DN $\gamma\delta$ thymocytes that were generated in WT $\gamma\delta$ TCR Tg, Lck^{+/-} $\gamma\delta$ TCR Tg and Fyn^{+/-} $\gamma\delta$ TCR Tg mice, we found that DN $\gamma\delta$ thymocytes in the three genotypes

Table 1. Percentage of Ki-67⁺ DN $\gamma\delta$ TCR⁺ thymocytes^a.

Genotype	% Ki-67 ⁺ DN $\gamma\delta$ TCR ⁺
WT $\gamma\delta$ TCR Tg	32.6±1.2
Lck ^{+/-} $\gamma\delta$ TCR Tg	43.7±6.0**
Fyn ^{+/-} $\gamma\delta$ TCR Tg	32.3±1.8

^aKi-67 expression marks cells in late G₁ phase through mitosis and is used as marker of active cell cycling.

***p*≤0.01.

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expressed different levels of the $\gamma\delta$ TCR but equivalent levels of CD5. Specifically, Lck^{+/-} $\gamma\delta$ thymocytes expressed significantly higher levels of the $\gamma\delta$ TCR than WT $\gamma\delta$ thymocytes, whereas Fyn^{+/-} $\gamma\delta$ thymocytes expressed significantly lower levels of the $\gamma\delta$ TCR than WT $\gamma\delta$ thymocytes (Fig. 4A). These data suggested that reducing Lck expression levels weakened $\gamma\delta$ TCR signal strength to the extent that immature thymocytes expressing relatively high levels of the $\gamma\delta$ TCR adopted the $\gamma\delta$ fate. Conversely, reducing Fyn expression levels augmented $\gamma\delta$ TCR

signal strength to where immature thymocytes expressing relatively low levels of the $\gamma\delta$ TCR adopted the $\gamma\delta$ fate.

To investigate the effects of reducing the levels of Lck or Fyn on the maturation of $\gamma\delta$ T cells in the thymus and their subsequent ability to migrate to the periphery, we enumerated DN $\gamma\delta$ T cells in the LNs of WT $\gamma\delta$ TCR Tg, Lck^{+/-} $\gamma\delta$ TCR Tg, and Fyn^{+/-} $\gamma\delta$ TCR Tg mice. Compared to WT $\gamma\delta$ TCR Tg mice, we observed a significant decrease in the number of DN $\gamma\delta$ T cells in Lck^{+/-} $\gamma\delta$ TCR Tg mice but not in the number of DN $\gamma\delta$ T cells in Fyn^{+/-} $\gamma\delta$ TCR Tg mice (Fig. 3C). Consistent with the lower numbers of peripheral $\gamma\delta$ T cells in Lck^{+/-} $\gamma\delta$ TCR Tg mice was the finding that there were fewer DN $\gamma\delta$ T cells in these mice that expressed CD24, a marker of recent thymic emigrant $\gamma\delta$ T cells [36], and that there were more cells that expressed CD44, a marker of activated cells, memory cells, and/or cells undergoing homeostatic proliferation [36–38] (Fig. 4B). Moreover, we found that the $\gamma\delta$ TCR and CD5 surface levels that were noted among the DN $\gamma\delta$ TCR⁺ thymocytes from the three genotypes were maintained on their respective peripheral $\gamma\delta$ T cells, with the exception that Lck^{+/-} $\gamma\delta$ T cells expressed significantly lower levels of CD5 than WT $\gamma\delta$ T cells (Fig. 4C). Therefore, although DN $\gamma\delta$ thymocytes

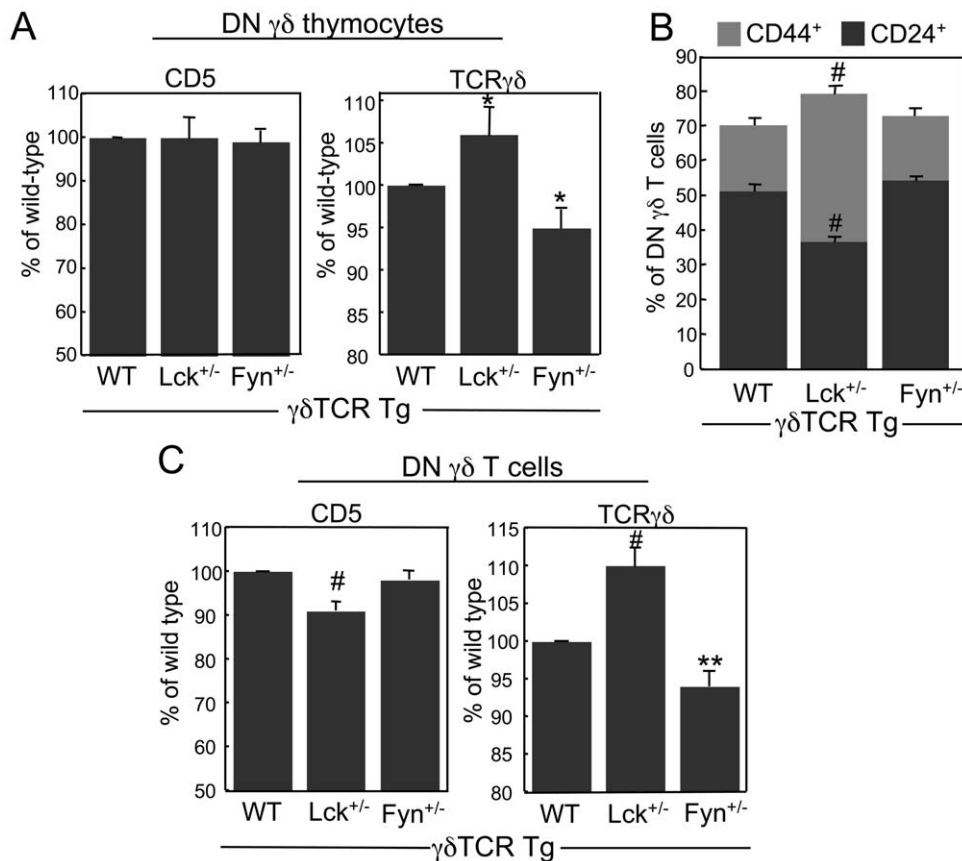


Figure 4. Phenotypic analysis of $\gamma\delta$ lineage cells from Lck^{+/-} $\gamma\delta$ TCR Tg and Fyn^{+/-} $\gamma\delta$ TCR Tg mice. A. Comparison of CD5 and TCR $\gamma\delta$ surface levels on DN $\gamma\delta$ thymocytes from WT $\gamma\delta$ TCR Tg, Lck^{+/-} $\gamma\delta$ TCR Tg and Fyn^{+/-} $\gamma\delta$ TCR Tg mice. MFIs of CD5 and TCR $\gamma\delta$ surface levels on DN $\gamma\delta$ thymocytes from heterozygous mice are presented as a percentage of the MFIs of CD5 and TCR $\gamma\delta$ surface levels on DN $\gamma\delta$ thymocytes from WT $\gamma\delta$ TCR Tg mice. Data represent at least 6 mice per genotype. B. Percentage of CD24⁺ and CD44⁺ $\gamma\delta$ T cells in WT $\gamma\delta$ TCR Tg, Lck^{+/-} $\gamma\delta$ TCR Tg and Fyn^{+/-} $\gamma\delta$ TCR Tg mice. Data represent at least 3 mice per genotype. C. Comparison of CD5 and TCR $\gamma\delta$ surface levels on DN $\gamma\delta$ T cells from the LNs of WT $\gamma\delta$ TCR Tg, Lck^{+/-} $\gamma\delta$ TCR Tg and Fyn^{+/-} $\gamma\delta$ TCR Tg mice. MFIs of CD5 and TCR $\gamma\delta$ surface levels on peripheral DN $\gamma\delta$ T cells from heterozygous mice are presented as a percentage of the MFIs of CD5 and TCR $\gamma\delta$ surface levels on peripheral DN $\gamma\delta$ T cells from WT $\gamma\delta$ TCR Tg mice. Data represent at least 5 mice per genotype. In A, B, and C, the bars represent mean \pm SEM. **p*≤0.05, ***p*≤0.01, #*p*≤0.001.

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were generated in $Lck^{+/-}$ $\gamma\delta$ TCR Tg mice in numbers comparable to WT $\gamma\delta$ TCR Tg mice, these mice had reduced numbers of mature DN $\gamma\delta$ T cells. These data indicated that a reduction in Lck levels but not Fyn levels affects the maturation and/or survival of thymic $\gamma\delta$ T cells.

Effect of Reducing Lck or Fyn Levels on $\gamma\delta$ T Cell Effector Fate and Function

$\gamma\delta$ T cell effector fate has been shown to segregate with expression of specific surface antigens, specifically $CD122^+$ and/or $CD27^+$ $\gamma\delta$ T cells preferentially produce $IFN\gamma$ [39,40], whereas $IL-23R^+$ $\gamma\delta$ T cells preferentially produce $IL-17$ [41–43]. Given these findings, we sought to determine whether reducing the levels of Lck or Fyn altered the ability of a $\gamma\delta$ T cell to become an $IL-17$ - and/or $IFN\gamma$ -producing effector cell. To accomplish this, we chose

to use our $\gamma\delta$ TCR Tg mouse model as it generates, on the WT background, 30-fold more DN $\gamma\delta$ T cells than non- $\gamma\delta$ TCR Tg mice [30]. This means that DN $\gamma\delta$ T cells can be analyzed without the concern that purification by positive selection using an anti-TCR $\gamma\delta$ mAb may crosslink the $\gamma\delta$ TCR and, in turn, pre-activate the $\gamma\delta$ T cell. First, we determined whether there were any differences in the percentages of $\gamma\delta$ T cells expressing $CD122$, $CD27$ or $IL-23R$ among WT $\gamma\delta$ TCR Tg, $Lck^{+/-}$ $\gamma\delta$ TCR Tg, and $Fyn^{+/-}$ $\gamma\delta$ TCR Tg mice. We found that both $Lck^{+/-}$ $\gamma\delta$ TCR Tg and $Fyn^{+/-}$ $\gamma\delta$ TCR Tg mice had similar percentages of $CD27^+$ and $CD27^-$ $\gamma\delta$ T cells as WT $\gamma\delta$ TCR Tg mice, but the percentages of $CD27^+$ cells co-expressing $CD122^+$ in both $Lck^{+/-}$ $\gamma\delta$ TCR Tg and $Fyn^{+/-}$ $\gamma\delta$ TCR Tg mice was reduced compared to WT $\gamma\delta$ TCR Tg mice (Fig. 5A). As $CD122$ expression by $\gamma\delta$ T cells is induced when the $\gamma\delta$ TCR interacts with its ligand in the

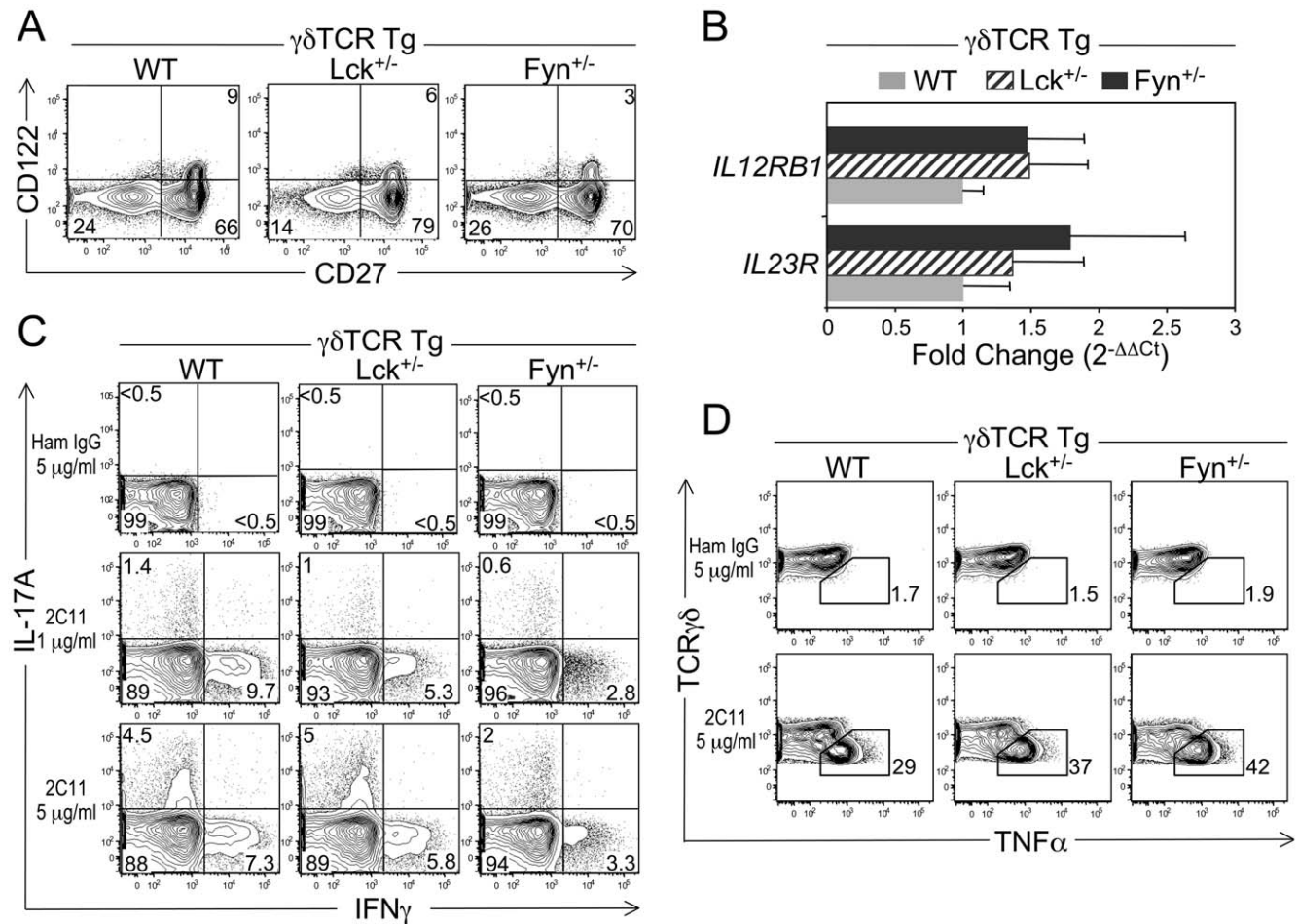


Figure 5. Effect of reducing Lck or Fyn levels on $\gamma\delta$ T cell effector fate and function. A. Dot plots showing representative $CD122$ versus $CD27$ staining profiles for DN $\gamma\delta$ TCR⁺ LN cells from WT $\gamma\delta$ TCR Tg, $Lck^{+/-}$ $\gamma\delta$ TCR Tg and $Fyn^{+/-}$ $\gamma\delta$ TCR Tg mice. Numbers in the quadrants represent percentage of cells in that quadrant. The percentage of $CD122^+$ DN $\gamma\delta$ T cells is significantly lower in $Fyn^{+/-}$ $\gamma\delta$ TCR Tg mice than in WT $\gamma\delta$ TCR Tg mice, $p \leq 0.05$. Data are representative of at least 6 mice per genotype. B. Quantitative real-time RT-PCR analysis of the relative transcript levels of *IL12RB1* and *IL23R* in purified peripheral DN $\gamma\delta$ T cells from WT $\gamma\delta$ TCR Tg, $Lck^{+/-}$ $\gamma\delta$ TCR Tg and $Fyn^{+/-}$ $\gamma\delta$ TCR Tg mice. Data are normalized to *GAPDH* and are presented as fold change over WT $\gamma\delta$ T cells (set to 1). Bars represent mean \pm SEM. Data represent 3 mice per genotype. C. Comparison of $IL-17$ and $IFN\gamma$ production by DN $\gamma\delta$ TCR⁺ LN cells from WT $\gamma\delta$ TCR Tg, $Lck^{+/-}$ $\gamma\delta$ TCR Tg and $Fyn^{+/-}$ $\gamma\delta$ TCR Tg mice. LN cells from the three genotypes were *in vitro* stimulated with 1 or 5 μ g/ml of immobilized anti-CD3 mAb or 5 μ g/ml of immobilized hamster IgG. 16 h later, cells were harvested and cytokine production was assayed by i.c. flow cytometric analysis. Dot plots show representative i.c. staining for $IFN\gamma$ versus $IL-17$ in gated DN $\gamma\delta$ TCR⁺ cells. Numbers in the quadrants represent percentage of cells in that quadrant. Data shown are representative of at least 3 mice per genotype. D. Comparison of the ability of DN $\gamma\delta$ T cells from WT $\gamma\delta$ TCR Tg, $Lck^{+/-}$ $\gamma\delta$ TCR Tg and $Fyn^{+/-}$ $\gamma\delta$ TCR Tg to produce $TNF\alpha$. Lymph node cells from each genotype were *in vitro* stimulated with 5 μ g/ml of immobilized anti-CD3 mAb or 5 μ g/ml of immobilized hamster IgG. 48 h later, cytokine production was measured by i.c. flow cytometry. Dot plots show representative staining for i.c. $TNF\alpha$ versus $TCR\gamma\delta$ in gated DN $\gamma\delta$ TCR⁺ cells. The percentage of $TNF\alpha$ -producing $\gamma\delta$ T cells for each genotype is shown. Data are representative of at least 4 mice per genotype. doi:10.1371/journal.pone.0008899.g005

thymus [39], these results suggested that the selection and/or survival of CD122⁺ $\gamma\delta$ T cells is impaired when Lck or Fyn levels are reduced. To evaluate IL-23R expression among WT, Lck^{+/-}, and Fyn^{+/-} $\gamma\delta$ T cells, we performed quantitative real-time RT-PCR analysis to detect transcription of *IL12RB1* and *IL23R*, which encode the two subunits of the IL-23R [44]. No significant differences were noted in the relative amounts of *IL12RB1* and *IL23R* transcripts among WT, Lck^{+/-}, and Fyn^{+/-} $\gamma\delta$ T cells (Fig. 5B), indicating that the selection and/or survival of $\gamma\delta$ T cells with the potential to produce IL-17 is not affected when Lck or Fyn levels are reduced.

Next, we assessed cytokine production by WT, Lck^{+/-}, and Fyn^{+/-} $\gamma\delta$ T cells following CD3 crosslinking. Interestingly, we found that the percentages of WT, Lck^{+/-} and Fyn^{+/-} $\gamma\delta$ T cells producing IFN γ at 16 h were equivalent to the percentages of CD122⁺ CD27⁺ $\gamma\delta$ T cells in each mouse (Fig. 5A and C). It is also important to note that the level of IFN γ production, as measured by MFI, was 2 to 3-fold less in Lck^{+/-} and Fyn^{+/-} IFN γ ⁺ $\gamma\delta$ T cells than in WT IFN γ ⁺ $\gamma\delta$ T cells (Fig. 5C and data not shown). Moreover, when we compared the ability of WT, Lck^{+/-} and Fyn^{+/-} $\gamma\delta$ T cells to differentiate into IL-17-producing cells at 16 h, we detected considerably fewer Fyn^{+/-} IL-17⁺ $\gamma\delta$ T cells than Lck^{+/-} or WT IL-17⁺ $\gamma\delta$ T cells (Fig. 5C). Taken together, these data indicated that reducing the levels of Fyn impacts the function of $\gamma\delta$ T cells that have the potential to become either IL-17- or IFN γ -producing effector cells, while reducing the levels of Lck only impacts the function of $\gamma\delta$ T cells that have the potential to become IFN γ -producing effector cells.

Because Lck^{+/-} and Fyn^{+/-} $\gamma\delta$ T cells do not efficiently produce IFN γ , it was of interest to determine whether reducing Lck or Fyn levels also affected the ability of $\gamma\delta$ T cells to produce other cytokines. To test this, we evaluated TNF α production by $\gamma\delta$ T cells from Lck^{+/-} $\gamma\delta$ TCR Tg and Fyn^{+/-} $\gamma\delta$ TCR Tg mice, since $\gamma\delta$ T cells, including those that produce IFN γ , have been shown to produce this cytokine [40]. As shown in Fig. 5D, we found that more $\gamma\delta$ T cells from Lck^{+/-} $\gamma\delta$ TCR Tg and Fyn^{+/-} $\gamma\delta$ TCR Tg mice than from WT $\gamma\delta$ TCR Tg mice were producing TNF α . These data indicated that reducing Lck or Fyn levels does not impair the ability of $\gamma\delta$ T cells to produce TNF α and suggested that the TCR signals required to activate the genetic program for IFN γ production are different than those for TNF α production.

Discussion

Since SFKs have both positive and negative roles in receptor signaling, it has been postulated that these kinases function more like rheostats than on/off switches [45]. Our data support this idea, as changes in the cellular levels of Lck or Fyn at different stages in the life of a $\gamma\delta$ lineage cell affected the strength of the $\gamma\delta$ TCR signaling response and, in turn, affected $\alpha\beta/\gamma\delta$ lineage commitment, $\gamma\delta$ T cell maturation and $\gamma\delta$ T effector cell differentiation.

The expression levels of Lck and Fyn change during T cell development and maturation. Immature thymocytes (i.e., DN2 thymocytes), which have the potential to become either $\alpha\beta$ or $\gamma\delta$ lineage cells [26,27], expressed relatively high levels of both Lck and Fyn. In thymic $\gamma\delta$ lineage cells, these high levels of Lck and Fyn were maintained and, not until the $\gamma\delta$ lineage cells were exported from the thymus, did their Lck and Fyn expression levels decrease. However, in immature $\alpha\beta$ lineage cells, Lck and Fyn expression levels dramatically declined and, at the DP stage, their levels of Lck and Fyn were extremely low. The low SFK expression in DP thymocytes has also been reported by Olszowy et al. [46], who used quantitative Western blot analysis to measure

Lck and Fyn protein levels in thymocyte subsets. Interestingly, Lck and Fyn levels were increased in TCR^{hi} DP (data not shown) and SP thymocytes compared to DP thymocytes, suggesting that positive selection upregulated both Lck and Fyn expression. It is important to note that mature SP thymocytes, after leaving the thymus, downregulated Lck and Fyn expression levels, but not to the levels observed for DP thymocytes nor to the extent observed between thymic and peripheral $\gamma\delta$ T cells.

To investigate the importance of the quantitative difference in Lck and Fyn expression levels between thymic and peripheral $\gamma\delta$ T cells, we used Lck^{+/-} and Fyn^{+/-} mice to study the effect of reducing Lck or Fyn expression levels on $\gamma\delta$ T cell development and function. We chose to reduce, as opposed to eliminate, Lck and Fyn expression levels to prevent any compensatory action that one SFK may exhibit in the absence of the other. Although Fyn levels were reduced by 50% in $\alpha\beta$ lineage cells from Fyn^{+/-} mice, they were only reduced by 40% in $\gamma\delta$ lineage cells from the same mice. There are two possible explanations, which are not mutually exclusive, for why Fyn levels were not reduced to the expected 50% in $\gamma\delta$ lineage cells from Fyn^{+/-} mice. First, the relatively high levels of Fyn in Fyn^{+/-} $\gamma\delta$ thymocytes may be a result of selection, where only cells with high levels of Fyn survive and continue to mature. The second possibility is that the high Fyn levels in $\gamma\delta$ lineage cells reflect high Fyn levels in a precursor population, such as DN2 thymocytes, which have the developmental potential to give rise to $\alpha\beta$ and $\gamma\delta$ lineage cells [26,27]. Indeed, we found that Fyn expression was only reduced by ~15% in DN2 thymocytes from Fyn^{+/-} mice. Therefore, even though we cannot rule out selection of $\gamma\delta$ thymocytes with high levels of Fyn, it is conceivable that the relatively high levels of Fyn in $\gamma\delta$ thymocytes from Fyn^{+/-} mice may be a direct result of the high levels of Fyn in Fyn^{+/-} DN2 thymocytes.

The high level of Fyn in thymic precursors highlights the importance of Fyn activity during an early stage of T cell development. As surface TCR complexes are not expressed at this stage, it is possible that Fyn is required for signaling through other receptors. One such receptor may be the IL-7 receptor (IL-7R), as Fyn has been shown to be recruited to this receptor [47,48]. Given this association and that DN2 thymocytes require IL-7R expression and signaling for their survival and proliferation [49–53], it is possible that relatively high levels of Fyn are required for proper IL-7R signaling at this stage.

The first stage in T cell development where we observed regulation of $\gamma\delta$ TCR signal strength by Lck and Fyn is during $\alpha\beta/\gamma\delta$ lineage commitment. We and others have previously demonstrated that TCR signal strength influences the $\alpha\beta/\gamma\delta$ lineage decision, with a strong signal favoring $\gamma\delta$ lineage commitment and a weak signal favoring $\alpha\beta$ lineage commitment [29,33,54]. By fixing the specificity of the $\gamma\delta$ TCR, we were able to detect changes in the $\gamma\delta$ TCR signal response that were not apparent with a polyclonal $\gamma\delta$ TCR repertoire. Reducing the expression of Lck weakened $\gamma\delta$ TCR signal strength and resulted in a striking increase in the percentage and number of DP thymocytes in Lck^{+/-} $\gamma\delta$ TCR Tg mice compared to WT $\gamma\delta$ TCR Tg mice. Moreover, weakening of the $\gamma\delta$ TCR signal was confirmed by the finding that DN $\gamma\delta$ thymocytes from Lck^{+/-} $\gamma\delta$ TCR Tg mice expressed higher levels of the $\gamma\delta$ TCR than WT DN $\gamma\delta$ thymocytes. Together, these results indicated that relatively high levels of Lck are required to achieve the appropriate TCR signal response to support the $\gamma\delta$ lineage choice. Conversely, reducing Fyn expression levels strengthened the $\gamma\delta$ TCR signaling response, as evidenced by the decrease, albeit not significant, in the number of DP thymocytes and the significant decrease in $\gamma\delta$ TCR surface expression on DN $\gamma\delta$ thymocytes from Fyn^{+/-}

$\gamma\delta$ TCR Tg mice. In contrast to the results of our previous study, in which $\gamma\delta$ T cell fate was favored over $\alpha\beta$ T cell fate when $\gamma\delta$ TCR signal strength was augmented [29], reducing Fyn expression had modest effects on the generation of $\alpha\beta$ and $\gamma\delta$ lineage cells. This difference may be attributed to the fact that Fyn expression was reduced by 35% in $\gamma\delta$ thymocytes from Fyn^{+/-} $\gamma\delta$ TCR Tg mice (data not shown), suggesting that immature DN thymocytes expressing low levels of Fyn were unable to survive and develop into $\alpha\beta$ or $\gamma\delta$ lineage cells.

Our data also demonstrated that the alterations in $\gamma\delta$ TCR signal strength by reducing Lck or Fyn expression levels affected $\gamma\delta$ T cell maturation in the thymus. When $\gamma\delta$ TCR signal strength was weakened by reducing Lck levels, there was a significant decrease in the number of DN $\gamma\delta$ T cells in LNs despite normal numbers of $\gamma\delta$ lineage cells present in the thymus. In support of a maturation defect, the frequency of recent thymic emigrants (CD24⁺ DN $\gamma\delta$ TCR⁺) was reduced in the LNs of Lck^{+/-} $\gamma\delta$ TCR Tg mice. Because of the reduction in thymic output, Lck^{+/-} $\gamma\delta$ T cells seemingly underwent homeostatic proliferation in the periphery, evidenced by the increased frequency of $\gamma\delta$ T cells with an activated/memory phenotype (CD44⁺ DN $\gamma\delta$ TCR⁺). On the other hand, strengthening the $\gamma\delta$ TCR signal response by reducing Fyn levels had little effect on thymic maturation. These data not only demonstrate that signaling by the $\gamma\delta$ TCR is required following $\gamma\delta$ lineage commitment for $\gamma\delta$ thymocyte maturation, but also indicate that the cellular levels of Lck in $\gamma\delta$ thymocytes regulate the $\gamma\delta$ TCR signaling response at this developmental stage.

Unlike $\alpha\beta$ thymocytes, $\gamma\delta$ thymocytes do not need to encounter Ag to mature and emigrate to the periphery [39,55]. However, a recent study demonstrates that Ag encounter in the thymus by $\gamma\delta$ T cells controls their effector fate, with Ag-experienced $\gamma\delta$ T cells expressing CD122 and preferentially producing IFN γ and Ag-naïve $\gamma\delta$ T cells lacking CD122 expression and preferentially producing IL-17 [39]. In addition to CD122 expression, $\gamma\delta$ T cell effector fate has been shown to segregate with CD27 expression, in that CD27⁺ $\gamma\delta$ T cells produce IFN γ and CD27⁻ $\gamma\delta$ T cells produce IL-17 [40]. We found that either weakening or augmenting the $\gamma\delta$ TCR signal response had an impact on the generation and/or survival of CD122⁺ CD27⁺ $\gamma\delta$ T cells but not on the generation and/or survival of CD122⁻ CD27⁺ $\gamma\delta$ T cells. Unfortunately, we do not know whether the defect lies in CD122⁺ $\gamma\delta$ T cell selection, survival or both, as very few (<0.5%) CD122⁺ $\gamma\delta$ T cells are detected in the thymus of WT $\gamma\delta$ TCR Tg, Lck^{+/-} $\gamma\delta$ TCR Tg, and Fyn^{+/-} $\gamma\delta$ TCR Tg mice (data not shown). Since V γ 6/V δ 1⁺ $\gamma\delta$ T cells are normally generated in the fetal thymus [28], the paucity of CD122⁺ $\gamma\delta$ thymocytes in WT $\gamma\delta$ TCR Tg mice may be due to decreased or negligible expression of the ligand for the V γ 6/V δ 1 $\gamma\delta$ TCR in the adult thymus. Nonetheless, we propose that weakening the $\gamma\delta$ TCR signal results in the generation of fewer CD122⁺ $\gamma\delta$ thymocytes following Ag encounter in the thymus. However, due to homeostatic proliferation in the periphery, the size of the CD122⁺ $\gamma\delta$ T cell pool is consequently increased. When the $\gamma\delta$ TCR signal is augmented, on the other hand, Ag encounter in the thymus may lead to negative selection and, in turn, fewer $\gamma\delta$ thymocytes and peripheral $\gamma\delta$ T cells expressing CD122. Surprisingly, in regards to $\gamma\delta$ T cells with the potential to produce IL-17, we found that neither weakening nor augmenting $\gamma\delta$ TCR signal strength had an impact on their generation and/or survival, based on the comparable expression of *IL23R* and *IL12RB1* in $\gamma\delta$ T cells from the three genotypes. These results indicate that $\gamma\delta$ T cells destined to become IL-17 producers, unlike those destined to become IFN γ producers, are not dependent on Lck- or Fyn-mediated signaling for their

generation and/or survival, and suggest that the choice to become an IFN γ effector versus an IL-17 effector is not a binary fate decision.

Consistent with their being multiple $\gamma\delta$ effector fates is our finding that, following TCR stimulation, 10% of WT $\gamma\delta$ T cells produce IFN γ , 5% produce IL-17 and 30% produce TNF α [a third of these also produce IFN γ , while none produce IL-17 (data not shown)]. Interestingly, the frequency of CD122⁺ $\gamma\delta$ T cells but not CD27⁺ $\gamma\delta$ T cells was found to be an indicator of IFN γ production by $\gamma\delta$ T cells, regardless of whether the stimulated $\gamma\delta$ T cells were from WT $\gamma\delta$ TCR Tg, Lck^{+/-} $\gamma\delta$ TCR Tg, and Fyn^{+/-} $\gamma\delta$ TCR Tg mice. While our finding is consistent with the findings of the recent study demonstrating that CD122 expression correlates with IFN γ production, it is not consistent with those of the other study demonstrating that CD27 expression correlates with IFN γ production. An explanation for the difference in results may be how, in each study, $\gamma\delta$ T cells were stimulated. In our study, as well as that of Jensen et al. [38], $\gamma\delta$ T cells were stimulated by crosslinking the TCR with anti-CD3 or anti-TCR δ mAbs. However, in the study of Ribot et al. [39], $\gamma\delta$ T cells were first activated and expanded in the presence of anti-CD3 mAb and IL-2 for four days prior to re-stimulation with the phorbol ester, phorbol 12-myristate 13-acetate, and ionomycin. These data, taken together, suggest that CD122⁺ $\gamma\delta$ T cells rapidly produce IFN γ following TCR activation, whereas CD27⁺ $\gamma\delta$ T cells require multiple days post TCR activation to differentiate into IFN γ -producing cells. Notably, both weakening and augmenting $\gamma\delta$ TCR signal strength affected the proficiency by which $\gamma\delta$ T cells produced IFN γ but not TNF α . This finding indicates that $\gamma\delta$ TCR signal strength regulates the quality of the cytokine effector response of stimulated $\gamma\delta$ T cells. Moreover, having both weak and strong TCR signals leading to a similar outcome is not novel in T cell biology, as for example it well known that DP thymocytes that receive TCR signals that are too weak or too strong undergo apoptosis [reviewed in 56].

In summary, we have demonstrated that the cellular levels of Lck or Fyn regulate the strength of the $\gamma\delta$ TCR signaling response. Specifically, we found that reducing Lck levels, thereby weakening $\gamma\delta$ TCR signal strength, had profound effects on $\alpha\beta$ / $\gamma\delta$ lineage commitment, $\gamma\delta$ thymocyte maturation, and the generation of IFN γ -producing effectors. In contrast, when Fyn levels were reduced, thus augmenting $\gamma\delta$ TCR signal strength, we observed defects in the generation of both IL-17- and IFN γ -producing effectors. Thus, this study has revealed that a relatively strong $\gamma\delta$ TCR signaling response is required following $\gamma\delta$ lineage commitment for $\gamma\delta$ T cell maturation and $\gamma\delta$ T effector cell differentiation.

Materials and Methods

Ethics Statement

All research involving animals has been conducted according to the relevant national and international guidelines with respect to husbandry, experimentation and welfare. Mouse protocols were approved by the SUNY Upstate Medical University Committee on the Humane Use of Animals.

Mice

C57BL/6 (wild-type or WT), B6.129S2-Lck^{tm1Mak}/J (Lck^{-/-}) and B6.129S7-Fyn^{tm1Sor}/J (Fyn^{-/-}) mice were originally purchased from The Jackson Laboratory (Bar Harbor, ME, USA). C57BL/6-V γ 6/V δ 1 $\gamma\delta$ TCR Tg mice ($\gamma\delta$ TCR Tg; line 134) [28] were kindly provided by P.E. Love (NICHD, Bethesda, MD, USA). Mice used in this study were bred and maintained in the Department of

Laboratory Animal Resources at SUNY Upstate Medical University in accordance with the specifications of the Association for Assessment and Accreditation of Laboratory Animal Care. All mice were sacrificed at 6–8 weeks of age.

Antibodies

Monoclonal antibodies (mAbs) used for flow cytometric analysis, $\gamma\delta$ T cell separation and $\gamma\delta$ T cell stimulation included anti-CD3 (145-2C11), anti-CD4 (RM4-5), anti-CD5 (53-7.3), anti-CD8 α (53-6.7), anti-CD8 β (53-5.8), anti-TCR $\gamma\delta$ (UC7-13D5), anti-TCR β (H57-597), anti-CD11b (M1/70), anti-CD16/CD32 (2.4G2), anti-CD19 (6D5), anti-IA^b (AF6-120.1), anti-CD24 (M1/69), anti-CD27 (LG.7F9), anti-CD44 (IM7), anti-CD122 (5H4), anti-NK1.1 (PK136), and anti-Ly6-G/Ly6-C (RB6-8C5), which were purchased from BD Pharmingen (San Jose, CA, USA), BioLegend (San Diego, CA, USA) or eBioscience (San Diego, CA, USA). mAbs used for intracellular (i.c.) flow cytometric analysis were Ki-67 (B56; BD Pharmingen), anti-Lck (3A5; Upstate Biotechnology, Billerica, MA, USA), anti-Fyn (FYN-59; BioLegend), anti-mouse IgG_{2b} (R12-3; BD Pharmingen), anti-IL-17A (TC11-18H10.1; BioLegend), anti-TNF α (MP6-XT22; BioLegend), and anti-IFN γ (XMG1.2; BD Pharmingen).

In Vitro Stimulation of $\gamma\delta$ T Cells

3×10^6 lymph node (LN) cells from WT, Lck^{+/-} and Fyn^{+/-} $\gamma\delta$ TCR Tg mice were resuspended in RPMI 1640 supplemented with non-essential amino acids, L-glutamine, HEPES, sodium pyruvate, and penicillin/streptomycin (all from Invitrogen, Carlsbad, CA, USA) in addition to 10% FBS (Mediatech, Inc., Manassas, VA, USA), plated onto 1 or 5 μ g/ml of immobilized anti-CD3 mAb or 5 μ g/ml of immobilized hamster isotype control, and then cultured for 16 or 48 h at 37°C. 5 h prior to fixation, permeabilization, and i.c. staining with mAbs against IL-17A, TNF α and/or IFN γ , cells were treated with Brefeldin A and Monensin (eBioscience).

Flow Cytometry

Flow cytometric analysis for surface antigens was performed by pre-incubating cells with the anti-CD16/CD32 mAb for at least 10 min to block non-specific binding of immunoglobulins to Fc receptors followed by staining with fluorochrome-conjugated Abs against the various surface antigens. I.c. staining for Ki-67 was performed according to the manufacturer's instructions (BD Pharmingen). I.c. staining for Lck and Fyn in addition to IL-17A, TNF α and IFN γ was performed by first fixing cells in a final concentration of 1.5% formaldehyde for 10 min at 37°C. Fixed cells were then stained for surface antigens, permeabilized with Perm/Wash Buffer (BD Pharmingen) for 20 min at 4°C, and then stained with mAbs against the appropriate proteins. The mAbs specific for IL-17A, TNF α and IFN γ were directly conjugated to a fluorochrome, while FITC-conjugated anti-mouse IgG_{2b} Ab was

used as a secondary reagent to detect the anti-Lck mAb or the anti-Fyn mAb. For all experiments, $0.1-2 \times 10^6$ cells were collected on a LSR II using FACSDiva software (BD Immunocytometry Systems, San Jose, CA, USA) and analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA). Dead cells were excluded from analysis based on forward and side scatter profiles.

Cell Separation

DN $\gamma\delta$ T cells were purified by negative selection from the LNs of WT $\gamma\delta$ TCR Tg, Lck^{+/-} $\gamma\delta$ TCR Tg and Fyn^{+/-} $\gamma\delta$ TCR Tg mice using the MACS magnetic bead separation system (Miltenyi Biotec, Auburn, CA, USA). LN cells were stained for 10 min at 4°C with a panel of FITC-labeled mAbs containing anti-CD19, anti-TCR β , anti-CD4, anti-CD8, anti-IA^b and anti-DX5 mAbs. Cells were washed, incubated with anti-FITC MACS beads for 15 min at 4°C, and then separated on an autoMACS cell separator, according to manufacturer's directions. The purity of the resulting DN $\gamma\delta$ T cell populations were typically $\geq 99\%$.

RT-PCR Analysis

RNA was extracted from purified DN $\gamma\delta$ T cells using the Qiagen RNeasy kit (Valencia, CA, USA). cDNA was then synthesized using Invitrogen's SuperScript[®] First-Strand Synthesis System. Quantitative real-time RT-PCR analysis was performed using a Bio-Rad iQTM5 Real-time PCR machine (Hercules, CA, USA) according to manufacturer's directions. All of the primer sets for the quantitative real-time RT-PCR analysis, which included *GAPDH*, *IL12RB1*, and *IL23R*, along with the SYBR Green PCR Master Mix were purchased from SABiosciences (Frederick, MD, USA).

Statistical Analysis

Data are presented as mean \pm SEM. Student's t-test was used for all statistical comparisons (Graph Pad Prism or Microsoft Excel software) except for the one evaluating Fyn levels in $\alpha\beta$ lineage cells, $\gamma\delta$ lineage cells and DN2 thymocytes from Fyn^{+/-} mice, in which a χ^2 test (Microsoft Excel software) was used. *p* values less than or equal to 0.05 were considered statistically significant.

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Author Contributions

Conceived and designed the experiments: RML SMH. Performed the experiments: RML SMH. Analyzed the data: RML SMH. Wrote the paper: RML SMH.

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