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Fyn Promotes Th17 Differentiation by Regulating the Kinetics of RORγt and Foxp3 Expression

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Th17 cells constitute a proinflammatory CD4+ T cell subset that is important for microbial clearance, but also are implicated as propagators of various autoimmune pathologies. Evidence suggests that Th17 cells share common progenitors with immunosuppressive CD4+ inducible regulatory T cells (T_REG) and that the developmental pathways of these two subsets are reciprocally regulated. In this study, we show evidence that the Src family tyrosine kinase Fyn helps regulate this Th17/T_REG balance. When placed under Th17-skewing conditions, CD4+ T cells from fyn−/− mice had decreased levels of IL-17, but increased expression of the T_REG transcription factor Foxp3. The defect in IL-17 expression occurred independently of the ectopic Foxp3 expression and correlated with a delay in retinoic acid-related orphan receptor γt upregulation and an inability to maintain normal STAT3 activation. Fyn-deficient Th17 cells also exhibited delayed upregulation of Il23r, Il21, Rora, and If4, as well as aberrant expression of Socs3, suggesting that Fyn may function upstream of a variety of molecular pathways that contribute to Th17 polarization. The fyn−/− mice had fewer IL-17+CD4+ T cells in the large intestinal lamina propria compared with littermate controls. Furthermore, after transfer of either wild-type or fyn−/− naive CD4+ T cells into Rag1−/− hosts, recipients receiving fyn−/− cells had fewer IL-17–producing T cells, indicating that Fyn may also regulate Th17 differentiation in vivo. These results identify Fyn as a possible novel regulator of the developmental balance between the Th17 cell and T_REG subsets. The Journal of Immunology, 2012, 188: 5247–5256.
9, 22). Conversely, Foxp3 is capable of binding the Th17 transcription factor RORγt and inhibiting its transcriptional activity (23). These reports indicate that the development of the Th17 and TREG lineages is a dynamic process that is ultimately determined by the amalgamation of often-opposing molecular signals. Such plasticity presumably provides the immune system a mechanism by which to rapidly react to changing requirements for either a proinflammatory or immunosuppressive response. Many other factors have been shown to modulate TREG versus Th17 development, such as retinoic acid (24), IFN regulatory factor (IRF) 4 (25), and the Akt/PI3K pathway (26, 27).

Although the Src family tyrosine kinases Fyn and Lck play a role in regulating TCR signals (28), much less is known about their function during Th differentiation. Lck appears to be required for the proper Th2, but not Th1, differentiation of naive CD4+ T cells (29, 30). In contrast, Fyn does not play an appreciable role in promoting either Th1 or Th2 development (31). In this report, we provide evidence that the tyrosine kinase Fyn may regulate the balance between TREG and Th17 differentiation by promoting RORγt upregulation, STAT3 activation, and Foxp3 downregulation in Th17-skewed CD4+ T cells. Our results therefore suggest a role for Fyn in modulating the homeostatic balance between the pro- and anti-inflammatory arms of the adaptive immune system.

Materials and Methods

Mice

All mice were on the C57BL/6 background, used at 6–12 wk of age, and housed in specific pathogen-free conditions in the Center of Comparative Medicine at the Feinberg School of Medicine at Northwestern University. The fyn<−/−> mice (32) specifically lack the FynT isoform of Fyn, which is predominately in regulating TCR signals (28), much less is known about their balance between TREG and Th17 differentiation by promoting either Th1 or Th2 development (31). In this report, we provide evidence that the tyrosine kinase Fyn may regulate the balance between TREG and Th17 differentiation by promoting RORγt upregulation, STAT3 activation, and Foxp3 downregulation in Th17-skewed CD4+ T cells. Our results therefore suggest a role for Fyn in modulating the homeostatic balance between the pro- and anti-inflammatory arms of the adaptive immune system.

Isolation and purification of primary CD4+ splenocytes

Splenoens were homogenized in wash buffer: DMEM supplemented with 5% calf serum, 200 mM t-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. RBCs were lysed using an NH4Cl solution. Bulk CD4+ cells, CD25-depleted CD4+ cells, or naive CD62L+ CD4+ cells were isolated using magnetic microbeads (Miltenyi Biotec). To isolate bulk CD4+ cells, RBC-lysed splenocytes were depleted with biotin-conjugated microbeads (Miltenyi Biotec); the resulting cells were routinely 98% CD4+CD62L+. Alternatively, RBC-lysed splenocytes were depleted with biotin-conjugated Abs against CD25, y8 TCR, CD8, CD11b, CD45R, and NK.1.1 (all from eBioscience) with streptavidin-conjugated microbeads to enrich for CD25-depleted CD4+ cells; the resulting cells were routinely ≥95% CD4+. To isolate CD62L−CD4+ cells, CD25-depleted CD4+ cells were further purified using anti-CD62L−conjugated microbeads (Miltenyi Biotec); the resulting cells were routinely ≥98% CD4+CD62L−.

Cell culture/Th subset skewing

Cultures were performed in 24-well plates (1 × 106 cells/well) with plate-bound 5 μg/ml anti-mouse CD28 (hybridoma 37.51) and 0.5 μg/ml anti-mouse TCRC (eBioscience) in T cell media: RPMI 1640 supplemented with 10% FBS (Foundation or Hyclone), 10 mM HEPES, 1 mM sodium pyruvate, 50 μM 2-ME, 1 mM t-glutamine, and 50 μg/ml gentamicin. Anti-mouse IFN-γ (11B11, 5μg/ml; BioXcell), anti-mouse IL-4 (XMG1.2, 5μg/ml; BioXcell), anti-mouse IL-12 (0.12 μg/ml; eBioscience), anti-mouse IL-2 (10 μg/ml; BD Pharmingen), mouse IL-6 (20 ng/ml), mouse IL-10 (20 ng/ml), mouse IL-17 (5 ng/ml; PeproTech), mouse IL-4 (10 ng/ml; PeproTech), mouse IL-23 (10 ng/ml; R&D Systems), and IL6656 (Cayman Chemical) were added as indicated. Specific Th-skewing conditions are shown in Supplemental Fig. 1A.

Retrovirus production and transduction

MIG (MSCV-IRES-GFP) constructs expressing RORγt or constitutively active STAT3 (MIG-RORγt and MIG-STAT3C, respectively) have been described previously (7, 8). MSCV-LTRmiR30-PIG (LMP) is a retrovector vector designed for the dual expression of GFP and short hairpin RNAs (shRNAs) (Open Biosystems). The LMP vector expressing an shRNA targeting Foxp3 (LMP-1066) has been described previously (23). Retroviruses were packaged in Phoenix cells, and virus-containing supernatant from these cultures was used for transduction of lymphocyte cultures. Briefly, cells were plated in nonskewing conditions with TCR/CD28 stimulation for 24 h, the culture media replaced with viral supernatant containing 8 μg/ml polybrene, and centrifuged at 2000 rpm for 90 min at 30°C on a tabletop centrifuge. Retroviral supernatant was then replaced with T cell media containing skewing cytokines and the cells cultured for an additional 4 to 5 d.

Cell staining and flow cytometry

For cytokine analysis, cells were stimulated for 4 h with 500 ng/ml ionomycin and 5 ng/ml PMA in the presence of a protein transport inhibitor (Monesin, eBioscience; or GolG1Stop, BD Biosciences). Cells were incubated with a FeR-blocker (2.4G2 hybridoma supernatant) before staining for surface markers in wash buffer. Fluorochrome-conjugated Annexin V and Abs against CD4 and CD25 were from eBioscience. For intracellular staining, cells were treated with either eBioscience (Foxp3, RORγt) or BD Biosciences (IL-17A, IFN-γ, IL-4, IL-2) fixation/permeabilization reagents and stained with the indicated fluorochrome-conjugated Abs in Permeablization/Wash Buffer (eBioscience): anti-IL-2 (BD Pharmingen), anti-IL-17 (BD Pharmingen or eBioscience), and anti-Foxp3. -RORγt, –IFN-γ, and –IL-4 (all from eBioscience). Staining of phosphorylated STAT3 (Y705) was performed using BD Phosflow reagents, according to the manufacturer’s protocol (BD Biosciences). Samples were run on a FACS960 II (BD Biosciences) at the Northwestern University Interdepartmental Immunobiology Core and data analyzed using FlowJo software (Tree Star). 7-Aminoactinomycin D or LIVE/DEAD Fixable Dead Cell reagent (Invitrogen) was used as an indicator of cell viability. Side-scatter (width and height) and forward-scatter (width and height) plots were used to gate on singlet events prior to all subsequent analyses.

Quantitative real-time RT-PCR

RNA was isolated from 1–5 × 106 cells using TRIzol reagent (Invitrogen). RNA concentration and absorption 260/280 was determined by Nanodrop (Thermoscientific) at the Genetics Core Facility at the Feinberg School of Medicine at Northwestern University. cDNA was reverse-transcribed from total RNA using Superscript III (Invitrogen) and random hexamer primers. Quantitative real-time RT-PCR was performed on 15 ng cDNA in triplicate using SYBR Green Master Mix (Applied Biosystems) and an Applied Biosystems 7000 Sequence Detection System (Applied Biosystems). Relative expression was determined by the ΔΔ threshold cycle method of comparative quantitative analysis, using β-actin expression as an internal control. Primer sequences are listed in Supplemental Table I. Primers for Rorγt (7) and Rora (33) were described previously.

Isolation of lamina proprial lymphocytes

The cecum and colon were cleaned of adipose and mesenteric tissue, cut open lengthwise, rinsed with PBS, and cut into 2-inch segments. Epithelial cells were removed by sequential shaking in DTT- and EDTA-containing PBS solutions. The remaining tissue was digested at 37°C in T cell media containing 200 U/ml collagenase VIII (Sigma-Aldrich) and 150 μg/ml DNase I (Sigma-Aldrich), and lamina proprial lymphocytes were isolated by a 40/60% Percoll gradient. Isolated cells were stimulated for 4 h with ionomycin and PMA in the presence of a protein transport inhibitor, and analyzed by flow cytometry.

Adaptive transfer of CD45RBhighCD4+ T cells

CD45RBhighCD4+ cells were isolated from the spleen of wild-type (WT) or fyn<−/−> donors and injected through the retro-orbital route into Rag1<−/−> recipients. Briefly, whole spleen homogenates from donor mice were enriched for CD4+ T cells by negative depletion using Abs against CD8, CD11b, CD45R, and MHC class II, followed by removal of Ab-conjugated cells. CD45RBhighCD4+ cells were isolated using a BioMag Goat Anti Rat IgG magnetic beads (Qiagen). Viable singlet CD45RBhighCD4+CD25+ cells (≥25% of the CD4+CD25+ subset with the highest CD45RB expression) were purified from this CD4+ enriched population by FACSort on a MoFlo High-Speed Sorter (Beckman Coulter) at the Robert H. Lurie Comprehensive Cancer Center Flow Cytometry Facility at Northwestern University. A total of 0.4 × 106 cells in 100 μl PBS was injected into age-matched Rag1<−/−> hosts. To assess cytokine expression by donor CD4+ T cells, indicated tissues from the indicated recipients were stimulated with ionomycin/PMA in the presence of a protein transport inhibitor, and IFN-γ and IL-17 production was assessed by intracellular staining and flow cytometry.
**Results**

**fyn** \(^{-/-}\) CD4\(^+\) T cells fail to polarize normally to the Th17 lineage

To assess the ability of **fyn** \(^{-/-}\) CD4\(^+\) cells to polarize toward the Th17 lineage, CD4\(^+\) splenocytes were isolated from WT or **fyn** \(^{-/-}\) mice and cultured in media containing TGF-\(\beta\) and IL-6 (Th17-skewing conditions; Supplemental Fig. 1A). WT CD4\(^+\) T cells produced high levels of IL-17 under these conditions, whereas **fyn** \(^{-/-}\) CD4\(^+\) T cells showed a marked reduction in IL-17 expression (Fig. 1A, 1B). Furthermore, **fyn** \(^{-/-}\) CD4\(^+\) T cells under Th17-polarizing conditions expressed high levels of Foxp3, a transcription factor associated with TREG (Fig. 1A, 1B). To preclude the effect of contamination by previously activated or memory CD4\(^+\) T cells, we performed a more stringent purification of naive CD62L\(^{-/-}\)CD4\(^+\) T cells, and obtained similar results (Fig. 1C). Like WT controls, **fyn** \(^{-/-}\) Th17 cultures produced negligible levels of IFN-\(\gamma\) and IL-4 (data not shown), suggesting that the defect in Th17 polarization is not due to an aberrant presence of Th1 and Th2 cytokines that inhibit Th17 differentiation.

**fyn** \(^{-/-}\) CD4\(^+\) T cells polarized normally under Th1-, Th2-, and TREG-skewing conditions, suggesting that **fyn** \(^{-/-}\) CD4\(^+\) T cells have a specific defect in polarization toward the Th17 lineage (Fig. 1D). **fyn** \(^{-/-}\) mice have normal percentages of CD25\(^+\)Foxp3\(^+\) CD4\(^+\) cells in the thymus and spleen, suggesting that natural TREG development is unaffected in the absence of Fyn (data not shown). Using Helios as a marker to distinguish natural from inducible TREG (34), we analyzed the percentage of Foxp3\(^+\) natural (Helios\(^+\)) and inducible (Helios\(^-\)) TREG in steady-state **fyn** \(^{-/-}\) and littermate control mice and found comparable levels of both cell populations in the gut, spleen, and thymus (data not shown).

**Lck**, another Src kinase family member with important roles in T cell differentiation and function, plays a role in Th2 differentiation (29, 30). However, **lck** \(^{-/-}\) CD4\(^+\) T cells expressed normal levels of IL-17 and Foxp3 under Th17-skewing conditions (Supplemental Fig. 1B).

We considered the defect in Th17 differentiation of **fyn** \(^{-/-}\) CD4\(^+\) cells could result from a nonspecific alteration in T cell development caused during the genetic deletion of fyn. Therefore, we treated WT Th17 cultures with SU6656, an Src kinase inhibitor that exhibits a 40-fold greater selectivity for Fyn than for Lck (36). SU6656 treatment of WT Th17 cultures caused a dose-dependent increase in Foxp3 and decrease in IL-17 expression (Fig. 1E). The inhibitor had no effect on IFN-\(\gamma\) production by WT Th1 cultures (Fig. 1F), suggesting that SU6656 does not have a general inhibitory effect on T cells at the concentrations tested. SU6656 treatment did not affect IL-17 or Foxp3 expression in **fyn** \(^{-/-}\) Th17 cultures (data not shown), further suggesting that SU6656’s effect on IL-17 and Foxp3 expression in WT Th17 cells is due to specific inhibition of Fyn. Therefore, both the pharmacological inhibition and the genetic deletion of Fyn support the concept that Fyn plays a specific role in Th17 differentiation.

We next considered whether differences in TGF-\(\beta\) or IL-6 signaling might contribute to the defective Th17 polarization of **fyn** \(^{-/-}\) CD4\(^+\) T cells. To address this question, we performed a titration of IL-6 and TGF-\(\beta\) in the Th17 skewing of WT and **fyn** \(^{-/-}\) CD25-depleted CD4\(^+\) splenocytes. At every concentration of IL-6 and TGF-\(\beta\) tested, the percentage of cells producing Foxp3 was higher in **fyn** \(^{-/-}\) Th17 cells than in WT Th17 cells (Supplemental Fig. 1C), suggesting that **fyn** \(^{-/-}\) CD4\(^+\) T cells have an increased propensity to express Foxp3 under Th17-polarizing conditions. Furthermore, **fyn** \(^{-/-}\) Th17 cells had lower IL-17 expression compared with WT cells at every concentration of TGF-\(\beta\).
and IL-6 tested (Supplemental Fig. 1D). These defects in Foxp3 and IL-17 expression did not appear to be mediated by changes in the expression of either the IL-6 or TGF-β receptor, which was comparable between WT and fyn−/− Th17 cells at both early and late time points of in vitro skewing (Supplemental Fig. 1E).

A transient defect in STAT3 activation contributes to decreased IL-17 expression by fyn−/− Th17 cells

Although the expression of the IL-6R was comparable between WT and fyn−/− CD4+ T cells, it has been previously reported that Fyn and other Src family members can bind and enhance the activity of STAT3 (37, 38), a downstream mediator of the IL-6R and an important activator of IL-17 and RORγt expression (8, 9). Therefore, we used a flow cytometry-based assay to quantify the level of STAT3 activation, as indicated by phosphorylation at tyrosine 705 (Y705) (39). STAT3 was rapidly activated in response to Th17-skewing cytokines, and STAT3 activation in WT and fyn−/− CD4+ T cells was comparable during the very early stages of Th17 polarization (Fig. 2A). However, fyn−/− Th17 cells exhibited a transient defect in STAT3 activation during the mid-late phase (days 1–3) of the in vitro-polarization period (Fig. 2B). This also correlated with increased Socs3 mRNA in the mutant, which may contribute to the attenuation of STAT3 signaling (Fig. 3B). These defects were later reversed (day 4), at which point STAT3 activation in fyn−/− Th17 cells was equal to or greater than that found in WT Th17 cells. These results suggest that Fyn is transiently required to maintain STAT3 activation during the course of Th17 differentiation.

The proinflammatory cytokine IL-21 is produced by Th17 cells and also signals through a STAT3-dependent mechanism. In combination with TGF-β, IL-21 initiates an alternative pathway of Th17 differentiation in naive CD4+ T cells (21, 40). Defective STAT3 activation in fyn−/− CD4+ T cells skewed with TGF-β and IL-6 prompted us to ask whether fyn−/− CD4+ T cells also had a defect in Th17 polarization in response to TGF-β and IL-21. Indeed, under these conditions, fyn−/− CD4+ T cells exhibited a marked reduction in IL-17 production and increase in Foxp3 expression (Fig. 2C, right panels). Therefore, fyn−/− CD4+ cells fail to respond normally to an alternative Th17-skewing condition, which also requires STAT3 activity but is independent of IL-6R signaling.

STAT3 activation is necessary, though not sufficient, to drive optimal IL-17 expression in naive CD4+ T cells (8). The defective STAT3 activation observed in fyn−/− Th17 cells (Fig. 2B) led us to hypothesize that Fyn is needed to maintain sufficient STAT3 activity to drive IL-17 expression. We therefore transduced WT or fyn−/− CD4+ T cells with a retrovirus encoding STAT3C prior to the initiation of Th17 skewing (Fig. 2D). The introduction of exogenous STAT3 activity into fyn−/− Th17 restored IL-17 production to WT levels, suggesting that the Fyn deficiency deregulates IL-17 expression in Th17 cells by selectively disrupting normal STAT3 activation.

STAT3 transduction was also able to partially repress the aberrant Foxp3 expression in fyn−/− Th17 cells (Fig. 2D). These results are in agreement with previous reports that STAT3 is the mediator of IL-6–dependent inhibition of Foxp3 expression (9, 22) and suggest that the transient defect in STAT3 activation may...
contribute not only to defective IL-17 expression, but also to the aberrant Foxp3 expression in $fyn^{2/2}$ Th17 cells. Deficient IL-17 expression in $fyn^{2/2}$ Th17 cells is independent of aberrant Foxp3 expression. Foxp3 can bind and inhibit RORγt, disrupting RORγt-dependent expression of Th17-associated genes (23). Intracellular staining of WT and $fyn^{2/2}$ Th17 cells revealed that although $fyn^{2/2}$ Th17 cells have only a slight reduction in the percentage of RORγt-positive cells, a greater proportion of $fyn^{2/2}$ Th17 cells express the $T_{RESF}$-associated transcription factor Foxp3 (Fig. 4A, top panel). Because of the increased percentage of Foxp3+/RORγt+ double-positive cells present in the $fyn^{2/2}$ Th17 culture, we speculated that the abrogation of Th17-associated gene expression might be due to the previously demonstrated inhibitory function of Foxp3 (23). We therefore asked whether the ectopic Foxp3 expression observed in $fyn^{2/2}$ Th17 cells may be an additional cause of the decreased IL-17 production in these cells. To address this question, we compared the IL-17 production by WT and $fyn^{2/2}$ Th17 cells that were RORγt single positive (SP) or RORγt/Foxp3-double positive (DP) (Fig. 4A). This analysis revealed that IL-17 expression by $fyn^{2/2}$ Th17 cells was defective in the RORγtSP subset as well as the RORγt/Foxp3 DP subset (Fig. 4A, bottom panel). Because the RORγt SP population comprises the majority of both the WT and $fyn^{2/2}$ Th17 cultures, these results suggest that Foxp3-mediated inhibition of RORγt transcriptional activity is not the predominant mechanism by which IL-17 expression is decreased in $fyn^{2/2}$ Th17 cultures.

It remained possible that Foxp3-mediated inhibition of RORγt plays a role in abrogating IL-17 expression in the Foxp3+/RORγt+ population of $fyn^{2/2}$ Th17 cells. Therefore, we inhibited Foxp3 expression in both WT and $fyn^{2/2}$ Th17 cells using an shRNA construct targeting the mRNA transcript of Foxp3 (Fig. 4B). Prior to the initiation of Th17 skewing, WT and $fyn^{2/2}$ CD4+ splenocytes were transduced either with an empty GFP-expressing retroviral vector (LMP [control]) or one expressing mouse RORγt (MIG-RORγt) and, then placed under Th17-polarizing conditions. Foxp3 and IL-17 expression were analyzed by intracellular staining and flow cytometry. Plots are gated on viable singlet CD4+ GFP+ events. Results are representative of three experiments. Statistical significance between WT and $fyn^{2/2}$ means was determined by a two-tailed paired Student $t$ test: *$p \leq 0.05$, **$p \leq 0.001$. 

Deficient IL-17 expression in $fyn^{2/2}$ Th17 cells is independent of aberrant Foxp3 expression

FIGURE 3. Fyn regulates the kinetics of RORγt and Foxp3 expression during Th17 differentiation. (A) RORγt upregulation and Foxp3 downregulation are delayed in $fyn^{2/2}$ Th17 cells. Foxp3 and RORγt expression were analyzed by flow cytometry in WT and $fyn^{2/2}$ CD25-depleted CD4+ T cells at the indicated time points after the initiation of Th17 polarization. Plots are gated on viable singlet CD4+ events. Results are representative of three experiments. (B) Expression of Th17-associated genes in $fyn^{2/2}$ Th17 cells. Total RNA was isolated from WT and $fyn^{2/2}$ CD4+ cells after 48 h (left panel) or 5 d (right panel) under Th17-polarizing conditions, and gene expression was assessed by quantitative real-time RT-PCR. The data for each gene represent an average of at least three independent experiments and are depicted as a fold change over the expression of β-actin. Error bars denote 1 SD from the mean. Primer sequences are listed in Supplemental Table I. (C) Exogenous RORγt restores IL-17 expression in $fyn^{2/2}$ Th17 cells. WT or $fyn^{2/2}$ CD4+ splenocytes were transduced either with an empty GFP-expressing retroviral vector (MIG [control]) or one expressing mouse RORγt (MIG-RORγt [RORγt]) and, then placed under Th17-polarizing conditions. Foxp3 and IL-17 expression were analyzed by intracellular staining and flow cytometry. Plots are gated on viable singlet CD4+ GFP+ events. Results are representative of three experiments. Statistical significance between WT and $fyn^{2/2}$ means was determined by a two-tailed paired Student $t$ test: *$p \leq 0.05$, **$p \leq 0.001$. 

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of Foxp3 in \(\text{fyn}^{-/-}\) Th17 cells; however, suppression of Foxp3 had no effect on IL-17 production (Fig. 4B), suggesting that elevated Foxp3 expression in \(\text{fyn}^{-/-}\) Th17 cultures is not the cause of low IL-17 synthesis.

IL-2 signaling contributes to Foxp3 expression in T\(_{\text{REG}}\) (22, 41) and inhibits Th17 differentiation (42). \(\text{fyn}^{-/-}\) Th17 cells had similar or lower levels of IL-2 and CD25 expression, suggesting that the aberrant Foxp3 expression in \(\text{fyn}^{-/-}\) Th17 is not caused by changes in IL-2 signaling (Supplemental Fig. 2A, 2B). However, the aberrant Foxp3 expression in \(\text{fyn}^{-/-}\) Th17 cells was dependent on the presence of IL-2, as the addition of a neutralizing Ab against IL-2 (αIL-2) abrogated Foxp3 expression (Fig. 4C). In agreement with our observations using shRNA knockdown of Foxp3, the anti–IL-2–mediated repression of Foxp3 expression in \(\text{fyn}^{-/-}\) Th17 cells did not lead to an increase in IL-17 expression. This further suggested that the defect in IL-17 expression is not a downstream consequence of the increased Foxp3 expression (Fig. 4B, 4C).

Delayed RORγt upregulation contributes to defective IL-17 expression in \(\text{fyn}^{-/-}\) Th17 cells

The dynamic expression of RORγt and Foxp3 during the entire course of Th17 differentiation determines the Th17/T\(_{\text{REG}}\) fate decision (23, 43). Therefore, we next assessed RORγt and Foxp3 expression in WT and \(\text{fyn}^{-/-}\) Th17 cells at various times after the initiation of in vitro Th17 polarization (Fig. 3A). WT Th17 cells began to upregulate RORγt by the first day after the initiation of skewing (day 1) and were nearly all RORγt-positive by the second day (day 2) (Fig. 3A). By comparison, \(\text{fyn}^{-/-}\) Th17 cells exhibited a marked delay in the upregulation of RORγt, but by day 5, the percentage of RORγt-positive cells in both the WT and \(\text{fyn}^{-/-}\) Th17 cultures were similar.

We observed a large amount of transient Foxp3 expression in both WT and \(\text{fyn}^{-/-}\) Th17 cells during the course of the in vitro skewing process: both began to upregulate Foxp3 around day 1 after the initiation of Th17 skewing. By day 2, >50% of WT and \(\text{fyn}^{-/-}\) cells expressed Foxp3 (Fig. 3A). In WT Th17, the upregulation RORγt preceded that of Foxp3 (compare day 1 and day 2 expression); consequently, all cells transiently expressing Foxp3 also expressed RORγt, consistent with the notion that the majority of Th17 cells go through a RORγt/Foxp3+ stage (23). The transient Foxp3 expression was rapidly extinguished in WT Th17 cells; it was reduced 5-fold by day 3 and nearly undetectable by day 4. In contrast, the \(\text{fyn}^{-/-}\) Th17 culture retained a population of Foxp3+ cells that persisted despite the presence of the proinflammatory cytokine IL-6. Although \(\text{fyn}^{-/-}\) Th17 cells upregulated Foxp3 with kinetics similar to that of WT Th17, delayed RORγt upregulation in these cells led to an appreciable accumulation of Foxp3-SP and Foxp3/RORγ double-negative cells, populations not observed in large numbers within WT Th17 cultures at day 2. These changes were apparent at the mRNA level as well: \(\text{fyn}^{-/-}\) Th17 cells had a sustained elevation in aberrant Foxp3 (Foxp3) expression, whereas Rorc(γt) (RORγt) expression is decreased during the early stages but is normal by day 5 of Th17 skewing (Fig. 3B). Both WT and \(\text{fyn}^{-/-}\) CD25+ CD4+ splenocytes exhibited a similar lack of proliferation and high levels of apoptosis under Th17-skewing conditions, suggesting that the Foxp3+ cells in the \(\text{fyn}^{-/-}\) Th17 culture were de novo generated and not due to abnormal outgrowth of contaminating natural T\(_{\text{REG}}\) (Supplemental Fig. 2C).

To address whether the delayed upregulation of RORγt accounts for the defective IL-17 expression in \(\text{fyn}^{-/-}\) cells, WT and \(\text{fyn}^{-/-}\) CD4+ T cells were transduced with a retrovirus encoding RORγt prior to the initiation of Th17 skewing. The introduction of ex-
ogenuous RORγt was able to restore IL-17 to WT levels in fyn−/− Th17 cells, consistent with the notion that a defect in early RORγt expression may contribute to defective expression of IL-17 in fyn−/− Th17 cells (Fig. 3C). Notably, overexpression of RORγt was unable to suppress Foxp3 in the fyn−/− Th17 culture, in agreement with a previous report (23).

We next assessed whether the expression of additional Th17-associated genes was altered in fyn−/− Th17 cells. IL23R (IL-23) expression in Th17 cells requires STAT3 but not RORγt (8). The expression of IL23R was decreased in fyn−/− Th17 cells at 48 h after the initiation of Th17 skewing, but was comparable to WT by day 5 (Fig. 3B), suggesting that the late restoration of STAT3 activity is sufficient to restore IL23R expression in CD4+ T cells during later stages of Th17 differentiation. The expression of IL17 (IL-17) and IL23R (IL-23R) require both RORγt and STAT3 (8). As with IL23R, the expression of IL23R was decreased in fyn−/− Th17 cells at the early stages of Th17 differentiation, but comparable to WT levels by day 5 (Fig. 3B). Therefore, fyn−/− Th17 cells are able to eventually upregulate normal levels of IL23R despite the transient defect in RORγt and STAT3 expression/activation during the early stages of Th17 differentiation. Furthermore, fyn−/− Th17 cells retained responsiveness to IL-23 despite the relative decrease in IL23R expression; both WT and fyn−/− Th17 cells showed a similar fold increase in IL-17 and fold decrease in Foxp3 when skewing cultures were supplemented with IL-23 (Supplemental Fig. 3). In contrast, the expression of IL17 in fyn−/− Th17 cells had not normalized by day 5, suggesting that the late restoration of RORγt and STAT3 expression/activity is not sufficient to drive optimal IL17 expression. The regulation of IL-21 and IL-17 is also dependent on the transcription factor IRF4. As was noted with IL-21, IRF4 expression was reduced in fyn−/− Th17 cells at 48 h, but normalizes by day 5 (Fig. 3B). Similarly, Th17 development is partially dependent on RORγt; it too is reduced at 48 h in the fyn mutants, but expression recovers by day 5 (Fig. 3B). Therefore, Fyn appears to be necessary for the optimal upregulation of many intersecting molecular pathways that contribute to Th17 differentiation.

fyn−/− CD4+ T cells have decreased Th17 differentiation in vivo

We next examined whether Fyn regulates the expression of IL-17 by CD4+ T cells in vivo. The lamina propria of intestinal tissue is a highly active lymphoid microenvironment and is a reservoir for both Th17 cells (7) and T REG in vivo (44). We isolated lymphocytes from the large intestine lamina propria of either fyn−/− or littermate control (fyn+/− or fyn+/-) mice and measured the Foxp3 and IL-17 expression in CD4+ T cells. WT and fyn−/− had comparable lymphocyte cell numbers in the lamina propria (data not shown). In agreement with our in vitro skewing data, CD4+ T cells from fyn−/− mice expressed lower levels of IL-17 than CD4+ T cells from control mice (Fig. 5A, 5B, left panel). In contrast, the percentage of IFN-γ-producing CD4+ T cells was comparable between fyn−/− and control mice, suggesting that the gut CD4+ T cells from fyn−/− mice have a selective defect in IL-17 expression rather than a global inhibition of inflammatory cytokine production (Fig. 5B, right panel). We did not detect any differences in the percentage of Foxp3+ cells between fyn−/− and control mice (Fig. 5A). The Foxp3+ population is presumably comprised predominantly of T REG, which develop normally in the absence of Fyn, according to our in vitro studies. No significant difference in IL-17 levels was observed in the IL-17−producing CD4+TCRβ+ innate lymphoid tissue inducer population (data not shown), suggesting that Fyn selectively regulates IL-17 production in the T cell compartment.

Discussion

Increasing evidence suggests that the T REG and Th17 subsets may be induced from similar precursors by divergent developmental pathways. We provide evidence that the protein tyrosine kinase Fyn may regulate the reciprocal development of the Th17 and T REG lineages by orchestrating the temporal expression or activation of STAT3, RORγt, and Foxp3. fyn−/− CD4+ splenocytes placed under Th17-polarizing conditions did not fully upregulate the Th17-associated gene IL17. Instead, fyn−/− CD4+ T cells diverged into a T REG-like phenotype, expressing aberrant levels of Foxp3 and acquiring the ability to suppress the proliferation of naive CD4+ T cells in vitro (A. Ueda, unpublished observations, data not shown).

Our results suggest that the defect in IL-17 expression in fyn−/− Th17 cells occurs independently of the ectopic Foxp3 expression and that the RORγt expressed in the later stages of fyn−/− Th17 differentiation is not sufficient to promote the normal expression of IL-17. As previously reported (7, 51), WT cells rapidly upregulated RORγt when placed under Th17-skewing conditions. In contrast, fyn−/− Th17 cells exhibited a profound delay in RORγt upregulation (Fig. 3A); this early defect in RORγt expression may contribute to the later deficiency in IL-17 expression. Although Th17 differentiation requires RORγt expression, our results reveal that the proper timing of RORγt expression is crucial for the normal expression of Th17-associated genes. The kinetics of RORγt expression in fyn−/− Th17 cells (Fig. 3A, 3B) suggest that RORγt is important for promoting IL-17 expression during the early stages (i.e., days 1–3) of in vitro Th17 differentiation. The expression of two other transcription factors that play a role in Th17 differentiation, RORα (33) and IRF4 (25), were also reduced during early differentiation in the absence of Fyn (Fig. 3B). Although it remains unclear how Fyn promotes the expression of RORα and IRF4, the global effect of Fyn deletion on these transcription factors suggests that Fyn is an upstream mediator of a variety of the molecular cascades that contribute to Th17 differentiation.

The defect in RORγt expression in fyn−/− Th17 cells was most evident between days 1 and 3 (Fig. 3A); this corresponded to the
time points when a transient defect in STAT3 activation was also observed (Fig. 2A, 2B). We also note that suppressor of cytokine signaling 3, an important negative regulator of STAT3 activity, is elevated at 48 h (Fig. 3B). This may contribute further to a reduction in STAT3 function. We therefore hypothesize that Fyn is necessary to maintain normal STAT3 activation during Th17 differentiation and that a deregulation of STAT3 activation contributes to diminished RORγt and RORγt-dependent IL-17 expression in fyn−/− Th17 cells. The role of Fyn and other Src family kinases in STAT3 activation has been reported in cancers and cell lines (37, 38), and our current findings suggest that this pathway is also an important mediator of Th17 differentiation. During the early to middle stages (days 1–3) of the Th17 differentiation process, WT CD4+ T cells also upregulated Foxp3, which was extinguished as the differentiation process progressed (Fig. 3A). These results are in agreement with previous reports that Th17 cells transiently express Foxp3 during their development (23, 51). fyn−/− Th17, in contrast, were unable to efficiently quench Foxp3 expression (Fig. 3A). Because STAT3 mediates the IL-6–dependent downregulation of Foxp3 (22, 23, 52), these results suggest that Fyn may also help orchestrate proper Foxp3 expression during Th17 differentiation by sustaining STAT3 activation.

In addition to STAT3 activation, other mechanisms downstream of Fyn may be necessary to fully extinguish the transient Foxp3 expression that occurs during Th17 differentiation. One possible mechanism is the Akt/Pi3K signaling pathway, which is activated by Fyn (53) and is a negative regulator of Foxp3 expression (26, 27). Ablation of Pi3K/Akt activity has been shown to promote the upregulation of Foxp3 and a TREG-like gene expression profile in newly activated naive CD4+ T cells (27). Similarly, the forced expression of an active Akt construct impairs the TGF-β-induced upregulation of Foxp3 in naive CD4+ T cells (26). Akt negatively affects Foxp3 expression by phosphorylating and blocking the nuclear localization of the forkhead family transcription factors Foxo1 and Foxo3, positive regulators of Foxp3 gene expression (54, 55). Akt can also serve as a positive mediator of IL-17 expression (56). Indeed, we have also observed that Akt activation is decreased in fyn−/− CD4+ T cells relative to WT during the early stages of Th17 differentiation (A. Ueda, unpublished observations). Thus, Fyn may be an important upstream mediator of Akt’s ability to extinguish Foxp3 and promote IL-17 expression in Th17 cells. p38 MAPK, a downstream target of the PI3K/Akt pathway, has also been shown to posttranscriptionally promote IL-17 production in Th17 cells (57). Therefore, it is possible that the regulation of IL-17 by Fyn occurs at the level of protein translation as well as that of gene expression. The putative regulation of the Akt/Pi3K and MAPK pathways by Fyn during Th17 differentiation requires further studies.

Our results demonstrate that a precise temporal regulation of STAT3, RORγt, and Foxp3 expression is necessary for proper Th17 differentiation. fyn−/− CD4+ T cells had decreased IL-21 and IL-23R expression at 48 h after the initiation of Th17 skewing, but the expression of these genes was comparable to WT Th17 by day 5 of differentiation (Fig. 3A, 3B). This suggests that
the recovery of STAT3 (Fig. 2B) and RORγt (Fig. 3A) activity/ expression in fyn−/− Th17 cells during the late stages of differentiation are sufficient to drive the expression of Il12i and Il23r. In contrast, fyn−/− Th17 cells do not express WT levels of IL-17 even by day 5 (Figs. 1A, 3B). The precise role that early RORγt or STAT3 activity plays in promoting IL-17 expression remains to be determined; the temporal requirement may indicate a role in facilitating permissive histone or chromatin modifications at the IL-17 locus. IL-6 and TGF-β treatment of naïve CD4+ T cells induces permissive histone 3 hyperacetylation in the promoter and several conserved noncoding sequences within the IL-17 locus within 48 h (58). STAT3 and RORγt (33) have been shown to promote histone 3 acetylation at the promoter and conserved noncoding sequence 2, respectively, of the IL-17 locus in Th17-skewed cells. It is yet unclear whether Fyn may play a role in promoting permissive chromatin restructuring of the IL-17 locus during Th17 differentiation.

CD4+ T cells isolated from the gut of fyn−/− mice also had less IL-17 production than those obtained from control mice (Fig. 5A, 5B), corroborating our in vitro data showing that Fyn supports IL-17 expression in Th17 cells. naïve CD45RBhighCD4+ T cells by the Northwestern University Interdepartmental ImmunoBiology Flow Cytometry Facility and Cancer Center.

We thank all past and present members of the Stein and Zhou Laboratories of lymphokine activities and secreted proteins.

References


Supplementary Table I. Primer sequences for quantitative RT-PCR. The sequences of primers used in SYBR Green-based qRT-PCR analyses.

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Supplementary Figure 1. Fyn deletion alters TH17 polarization without affecting IL6 or TGFβ receptor expression.
Supplementary Figure 1. Fyn deletion alters T_{H}17 polarization without affecting IL6 or TGFβ receptor expression (continued).

a) Unless otherwise indicated, T_{H}17 and T_{REG} cultures were polarized in the indicated cytokine milieu for 5 days in the presence of plate-bound anti-TCRβ and anti-CD28. Cells under T_{H}1 and T_{H}2 conditions were removed from TCRβ/CD28 stimulation on day 4, and cultured for 2 additional days in fresh cytokine-containing media without TCRβ/CD28 stimulation.

b) CD4+ T-cells lacking Lck produce normal levels of Foxp3 and IL17 under T_{H}17-polarizing conditions. WT or lck^{-/-} CD4+ splenocytes were skewed in vitro under T_{H}17-polarizing conditions. IL17 and Foxp3 expression was analyzed by flow cytometry. Plots are gated on viable singlet CD4+ events. The quantitation of results from at least six experiments is shown.

c, d) fyn^{-/-} T_{H}17 cells express higher levels of Foxp3 and lower levels of IL17 despite changes in IL6 and TGFβ signal strength. IL6 or TGFβ concentrations were titrated in the T_{H}17 skewing of WT or fyn^{-/-} CD25-depleted CD4+ splenocytes. During the titration of one cytokine, the concentration of the other cytokine was kept constant at 20ng/ml or 1ng/ml for IL6 or TGFβ, respectively. After 5 days, Foxp3 (d) and IL17 (e) expression was analyzed by intracellular staining and flow cytometry in the viable singlet CD4+ gate. Results are representative of three experiments.

e) Expression of IL6 and TGFβ receptors is comparable in WT and fyn^{-/-} T_{H}17 cells. At 48 hours (left) and 5 days (right) after the initiation of T_{H}17 skewing, the expression of Il6ra and Tgfbr1 in WT and fyn^{-/-} CD4+ T-cells was determined by qRT-PCR. The average of three experiments is shown; error bars denote one standard deviation from the mean. n.s.: not significant, two-tailed paired Student’s t-test.
Supplementary Figure 2. *fyn*−/− T<sub>H</sub>17 cells do not have elevated expression of IL2 or the high-affinity IL2-receptor subunit CD25, and exhibit proliferation and survival comparable to WT T<sub>H</sub>17.

a, b) CD25-depleted CD4<sup>+</sup> splenocytes from WT or *fyn*−/− mice were skewed under T<sub>H</sub>17-polarizing conditions. At the indicated timepoints, cells were stimulated with ionomycin and PMA in the presence of a protein transport inhibitor, and IL2 expression was determined by intracellular staining (a). Alternatively, the surface expression of CD25 was assessed on unstimulated cells (b). Plots are gated on viable singlet CD4<sup>+</sup> events. Data is representative of 4 (IL2) or 3 (CD25) independent experiments. Statistical significance between WT and *fyn*−/− means was determined by a two-tailed paired Student’s t-test; **: p≤0.01.

c) MACS-purified CD25<sup>+</sup> or CD25<sup>−</sup> CD4<sup>+</sup> splenocytes were stained with CFSE and cultured under T<sub>H</sub>17-skewing conditions for 2 days. Apoptosis and cell death were assessed by AnnexinV and 7-AAD staining. Alternatively, viable cells were identified as those with little staining by LiveDead reagent (Invitrogen). Plots are gated on total singlet events. Data is representative of 2 independent experiments.
Supplementary Figure 3. *fyn*−/− *T*H17 cells retain IL23 responsiveness.
The *T*H17-skewing culture of WT and *fyn*−/− CD4+ T-cells was performed with or without the addition of exogenous IL23 (10ng/ml). IL17 and Foxp3 expression was determined by intracellular staining and flow cytometry. Plots are gated on viable singlet CD4+ events. Numbers above the bars represent the mean value from two experiments, and the error bars denote one standard deviation from the mean.