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

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_J Immunol_ 2012; 188:5247-5256; Prepublished online 25 April 2012; doi: 10.4049/jimmunol.1102241
http://www.jimmunol.org/content/188/11/5247
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 (TREG) 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/TREG balance. When placed under Th17-skewing conditions, CD4+ T cells from fyn±/− mice had decreased levels of IL-17, but increased expression of the TREG 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 Irf4, 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 TREG subsets. The Journal of Immunology, 2012, 188: 5247–5256.

A major hallmark of the adaptive immune system is the ability to mount specific responses to a variety of immunological challenges. This specificity is conferred in part through the divergent differentiation of CD4+ Th cell subsets, the distinct functions of which allow the immune system to tailor specific responses to pathogens. For example, development of the classically described Th1 or Th2 CD4+ T cell subsets promotes either a proinflammatory/cytotoxic or an Ab-mediated/humoral response, respectively (1).

Th17 cells constitute a third CD4+ T cell subset separate from the classical Th1 and Th2 lineages; this distinction is underscored by the unique immunological functions and developmental requirements of the Th17 cell lineage (2–4). Whereas the Th1 and Th2 subsets are regulated by the master transcription factors Tbet and Gata3, respectively (5, 6), Th17 cell differentiation depends on the transcription factor retinoic acid-related orphan receptor γt (RORγt) (7). The development of Th17 cells also requires the activity of STAT3, which mediates the efficient upregulation of RORγt and other Th17-associated genes such as IL-17 (8, 9). In addition to IL-17, Th17 cells also produce IL-21, IL-22, TNF-α, and GM-CSF; these cytokines mediate the various functions of the Th17 subset, which include microbial defense, leukocyte recruitment, and auto-crine-positive regulation of proinflammatory cytokine production (10). Although normal Th17-mediated inflammation is important for host defense against pathogens, it has also been implicated in a variety of autoimmune pathologies such as inflammatory bowel disease (11), multiple sclerosis (12), and rheumatoid arthritis (13, 14). Therefore, a tight regulation of the inflammatory properties of Th17 cells is necessary to use their beneficial immune functions while curtailing their pathogenic capabilities.

One mechanism by which the immune system attenuates inflammatory mechanisms is through an additional CD4+ T cell subset known as regulatory T cells (TREG). TREG are regulated by the signature transcription factor Foxp3 (15, 16) and suppress the proliferation and function of effector T cell subsets (17, 18). TREG are predominately divided into two subsets: the natural TREG that are derived from thymic precursors and the inducible TREG that develop from naive CD4+ precursors in peripheral lymphoid organs (19). Inducible TREG (henceforth referred to in this study as TREG) develop from the same naive CD4+ precursors as effector T cells, suggesting that an additional mechanism by which the adaptive immune system suppresses inflammation is diverting the development of CD4+ precursors from an inflammatory fate to an immunosuppressive one.

Both TREG and Th17 cells are induced by the cytokine TGF-β; TGF-β alone induces Foxp3 upregulation and skewing toward a TREG phenotype (15), whereas the additional presence of inflammatory cytokines such as IL-6 or IL-21 collaborate with TGF-β to initiate the development of Th17 cells (20, 21). The reciprocal development of the Th17 and TREG lineages is also reflected at the molecular level; STAT3, a transcription factor important for Th17 development, has been shown to inhibit the expression of Foxp3.
REGULATION OF Th17 DIFFERENTIATION BY Fyn

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 Th1 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 expressed by hematopoietic cells. Animal procedures conformed to American Association for Laboratory Animal Science standards and were approved by Northwestern University’s Institutional Animal Care and Use Committee.

Isolation and purification of primary CD4+ splenocytes

Spleens were homogenized in wash buffer: DMEM supplemented with 5% calf serum, 200 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. RBCs were lysed using an NH4Cl solution. Bulk CD4+ 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). Alternatively, RBC-lysed splenocytes were depleted with biotin-conjugated Abs against CD25, CD90, CD8, CD11b, CD45R, and NK1.1 (all from 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 L-glutamine, and 50 μg/ml gentamicin. Anti-mouse IFN-γ (11B11, 5 μg/ml; BioXCell), anti-mouse IL-4 (XM12, 5 μg/ml; BioXCell), anti-mouse IL-12 (10 μg/ml; eBioscience), anti-mouse IL-2 (10 μg/ml; BD Pharmingen), mouse IL-6 (20 ng/ml unless otherwise noted; PeproTech), human IL-21 (20 ng/ml; PeproTech), human IL-2 (10 ng/ml; R&D Systems), and SU6656 (Cay- man Chemical) were added as indicated. Specific Th-skewing conditions have been described previously (7, 8). MSCV-LTR-RmiR30-PG (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 Fopx3 (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 non-skewing conditions with TCR/CD28 stimulation for 24 h, the culture media replaced with viral supernatant containing 8 μg/ml polybrene, and centrifuged at 2500 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 (Monensin, eBioscience; or GolgiStop, 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 Permeabilization/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 Phosphowest reagents, according to the manufacturer’s protocol (BD Biosciences). Samples were run on an FACSCanto 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 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 absorbance 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 ΔΔct method of comparative quantification, using β-actin expression as an internal control. Primer sequences are listed in Supplemental Table I. Primers for Rora (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 using a BioMag Goat Anti-Rat IgG magnetic beads (Qiagen). Viable singlet CD45RBhighCD4+CD4+ cells (~25% of the CD4+CD25− subset with the highest CD45RB expression) were purified from this CD4+ enriched population by FACS 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 isolated four days after the indicated treatments 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.

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Results

\textit{fyn}\textsuperscript{−/−} CD4\textsuperscript{+} T cells fail to polarize normally to the Th17 lineage

To assess the ability of \textit{fyn}\textsuperscript{−/−} CD4\textsuperscript{+} cells to polarize toward the Th17 lineage, CD4\textsuperscript{+} splenocytes were isolated from WT or \textit{fyn}\textsuperscript{−/−} mice and cultured in media containing TGF-\(\beta\) and IL-6 (Th17-skewing conditions; Supplemental Fig. 1A). WT CD4\textsuperscript{+} T cells produced high levels of IL-17 under these conditions, whereas \textit{fyn}\textsuperscript{−/−} CD4\textsuperscript{+} T cells showed a marked reduction in IL-17 expression (Fig. 1A, 1B). Furthermore, \textit{fyn}\textsuperscript{−/−} CD4\textsuperscript{+} 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\textsuperscript{+} T cells, we performed a more stringent purification of naive CD62L\textsuperscript{+}CD4\textsuperscript{+} T cells, and obtained similar results (Fig. 1C). Like WT controls, \textit{fyn}\textsuperscript{−/−} 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.

\textit{fyn}\textsuperscript{−/−} CD4\textsuperscript{+} T cells polarized normally under Th1- and Th2-polarizing conditions, suggesting that \textit{fyn}\textsuperscript{−/−} CD4\textsuperscript{+} T cells have a specific defect in polarization toward the Th17 lineage (Fig. 1D). \textit{fyn}\textsuperscript{−/−} mice have normal percentages of CD25\textsuperscript{+}Foxp3\textsuperscript{+} CD4\textsuperscript{+} 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 Foxp3\textsuperscript{+} and inducible (Helios\textsuperscript{+}) TREG\textsuperscript{S} in steady-state \textit{fyn}\textsuperscript{−/−} 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, \textit{lck}\textsuperscript{−/−} CD4\textsuperscript{+} T cells (35) expressed normal levels of IL-17 and Foxp3 under Th17-skewing conditions (Supplemental Fig. 1B).

We considered that the defect in Th17 differentiation of \textit{fyn}\textsuperscript{−/−} CD4\textsuperscript{+} cells could result from a nonspecific alteration in T cell development caused during the genetic deletion of \textit{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 \textit{fyn}\textsuperscript{−/−} Th17 cultures (data not shown), further suggesting that SU6656\textsuperscript{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 \textit{fyn}\textsuperscript{−/−} CD4\textsuperscript{+} T cells. To address this question, we performed a titration of IL-6 and TGF-\(\beta\) in the Th17 skewing of WT and \textit{fyn}\textsuperscript{−/−} CD25-depleted CD4\textsuperscript{+} splenocytes. At every concentration of IL-6 and TGF-\(\beta\) tested, the percentage of cells producing Foxp3 was higher in \textit{fyn}\textsuperscript{−/−} Th17 cells than in WT Th17 cells (Supplemental Fig. 1C), suggesting that \textit{fyn}\textsuperscript{−/−} CD4\textsuperscript{+} T cells have an increased propensity to express Foxp3 under Th17-polarizing conditions. Furthermore, \textit{fyn}\textsuperscript{−/−} Th17 cells had lower IL-17 expression compared with WT cells at every concentration of TGF-\(\beta\).

\textbf{FIGURE 1.} \textit{fyn}\textsuperscript{−/−} CD4\textsuperscript{+} T cells fail to polarize normally to the Th17 lineage. (A and B) \textit{fyn}\textsuperscript{−/−} Th17 produce decreased amounts of IL-17 and increased levels of Foxp3. CD4\textsuperscript{+} splenocytes were polarized in vitro under Th17-skewing conditions. IL-17 and Foxp3 expression was assessed by intracellular staining and flow cytometry. (C) Fyn promotes Th17 polarization of naive CD4\textsuperscript{+} T cells. Naive CD62L\textsuperscript{+}CD4\textsuperscript{+} T cells were isolated from the spleens of WT or \textit{fyn}\textsuperscript{−/−} mice and skewed under Th17-polarizing conditions. Foxp3 and IL-17 expression was determined by intracellular staining and flow cytometry. Results are representative of two experiments. (D) \textit{fyn}\textsuperscript{−/−} CD4\textsuperscript{+} T cells polarize normally to the TREG-, Th1-, and Th2 lines. WT or \textit{fyn}\textsuperscript{−/−} CD4\textsuperscript{+} splenocytes were skewed in vitro under TREG, Th1-, or Th2-polarizing conditions. Foxp3 and cytokine expression in viable single CD4\textsuperscript{+} events was determined by intracellular staining and flow cytometry. Results are representative of two experiments. (E and F) Pharmacological inhibition of Fyn leads to a selective defect in Th17 differentiation. WT CD4\textsuperscript{+} splenocytes were skewed under Th1- (E) or Th1-polarizing (F) conditions in the presence of the indicated concentration of the Src family kinase inhibitor SU6656. IL-17, Foxp3, and IFN-\(\gamma\) expression was assessed by intracellular staining and flow cytometry in the viable single CD4\textsuperscript{+} gate. The data are represented as a ratio of the percentage of cells in treated versus untreated samples that express the indicated marker. Results are representative of three (E) or two (F) experiments. ***p ≤ 0.001, two-tailed unpaired Student \(t\) test for equal variances.
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 \( \text{fyn}^{-/-} \) CD4+ T 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 \( \text{fyn}^{-/-} \) Th17 cells

Although the expression of the IL-6R was comparable between WT and \( \text{fyn}^{-/-} \) 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 \( \text{fyn}^{-/-} \) CD4+ T cells was comparable during the very early stages of Th17 polarization (Fig. 2A). However, \( \text{fyn}^{-/-} \) 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 \( \text{fyn}^{-/-} \) 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.

![Figure 2](http://www.jimmunol.org/)

**FIGURE 2.** Transient defects in STAT3 activation contributes to decreased IL-17 expression in \( \text{fyn}^{-/-} \) Th17 cells. (A and B) \( \text{fyn}^{-/-} \) Th17 cells have a transient defect in STAT3 activation. STAT3 (phospho-Y705) was quantified by intracellular staining in WT (solid line), \( \text{fyn}^{-/-} \) (dotted line), and STAT3-deficient (filled) CD4+ T cells at the indicated time points after initiation of Th17 polarization. Plots are gated on viable singlet CD4+ events. Results are representative of two (A) or three (B) experiments. (C) \( \text{fyn}^{-/-} \) CD4+ T cells have reduced IL-17 and elevated Foxp3 expression in response to IL-21 and TGF-β. WT and \( \text{fyn}^{-/-} \) CD4+ splenocytes were cultured for 5 d in the presence of TCR/CD28 stimulation and TGF-β plus IL-21 or IL-6. Foxp3 and IL-17 expression was assessed by intracellular staining and flow cytometry. Plots are gated on viable singlet CD4+ events. Results are representative of three experiments. (D) STAT3C restores IL-17 expression and represses aberrant Foxp3 expression in \( \text{fyn}^{-/-} \) Th17 cells. WT or \( \text{fyn}^{-/-} \) CD4+ splenocytes were transduced either with an empty GFP-expressing retroviral vector (MIG [control]) or one expressing STAT3C (MIG-STAT3C [STAT3C]) and then placed under Th17-polarizing conditions. Foxp3 and IL-17 expression was analyzed by intracellular staining and flow cytometry. Plots are gated on viable singlet CD4+ GFP+ events. Results are representative of three experiments.
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 ROR\( \gamma \)t, disrupting ROR\( \gamma \)t-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 ROR\( \gamma \)t-positive cells, a greater proportion of \( fyn^{2/2} \) Th17 cells express the T\(_{REG}\)-associated transcription factor Foxp3 (Fig. 4A, top panel). Because of the increased percentage of Foxp3\(^{+}\)/ROR\( \gamma \)t\(^{+}\) 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 ROR\( \gamma \)t single positive (SP) or ROR\( \gamma \)t/Foxp3-double positive (DP) (Fig. 4A). This analysis revealed that IL-17 expression by \( fyn^{2/2} \) Th17 cells was defective in the ROR\( \gamma \)tSP subset as well as the ROR\( \gamma \)t/Foxp3 DP subset (Fig. 4A, bottom panel). Because the ROR\( \gamma \)t SP population comprises the majority of both the WT and \( fyn^{2/2} \) Th17 cultures, these results suggest that Foxp3-mediated inhibition of ROR\( \gamma \)t 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 ROR\( \gamma \)t plays a role in abrogating IL-17 expression in the Foxp3\(^{3/3}\)/ROR\( \gamma \)t\(^{+}\) 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 with either an empty GFP-expressing retroviral vector (LMP [control]) or one expressing mouse ROR\( \gamma \)t (MIG-ROR\( \gamma \)t) 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 single 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: \( *p < 0.05 \), \( **p < 0.001 \).
FIGURE 4. The defect in IL-17 expression by fyn−/− Th17 cells is independent of ectopic Foxp3 expression. (A) IL-17 production is reduced in fyn−/− RORγt SP Th17 cells. Foxp3, RORγt, and IL-17 expression was determined in WT and fyn−/− Th17 cultures by intracellular staining and flow cytometry. IL-17 expression was then determined in the Foxp3 SP, RORγt SP, Foxp3/RORγt double-negative, or DP populations. Results are representative of three experiments. (B) Foxp3 knockdown does not elevate IL-17 expression in fyn−/− Th17 cells. WT or fyn−/− CD4+ splenocytes were transduced either with an empty GFP-expressing retroviral vector (LMP [control]) or one expressing an shRNA targeting Foxp3 (LMP-1066 [Foxp3 KD]) and then placed under Th17-polarizing conditions. Foxp3 and IL-17 expression was analyzed by intracellular staining and flow cytometry. Plots are gated on viable singlet CD4+ GFP+ events. Results are representative of three experiments. (C) Inhibition of Foxp3 expression by IL-2 neutralization does not increase IL-17 expression in fyn−/− Th17 cells. WT or fyn−/− Th17 cultures were either not treated (NT) or supplemented with anti-mouse IL-2 (αIL-2). Foxp3 and IL-17 expression was analyzed by intracellular staining and flow cytometry. Plots are gated on viable singlet CD4+ events. Results are representative of four experiments.

We observed a large amount of transient Foxp3 expression in both WT and 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 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 fyn−/− Th17 culture retained a population of Foxp3+ cells that persisted despite the presence of the proinflammatory cytokine IL-6. Although 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/RORγt 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: 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 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 fyn−/− Th17 culture were de novo generated and not due to abnormal outgrowth of contaminating natural Treg (Supplemental Fig. 2C).

To address whether the delayed upregulation of RORγt accounts for the defective IL-17 expression in fyn−/− cells, WT and fyn−/− CD4+ T cells were transduced with a retrovirus encoding RORγt prior to the initiation of Th17 skewing. The introduction of ex-
ogenous 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. IL21 (IL-21) expression in Th17 cells requires STAT3 but not RORγt (8). The expression of IL21 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 IL21 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 Il21, 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/activity 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 TREG in vivo (44). We isolated lymphocytes from the large intestine lamina propria of either fyn−/− or littermate control (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 predominately of TREG, 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+TCRB+ innate lymphoid tissue inducer population (data not shown), suggesting that Fyn selectively regulates IL-17 production in the T cell compartment.

To determine whether Fyn is necessary in vivo for the differentiation of naive CD4+ T cells into the Th17 subset, we adoptively transferred CD45RBhighCD25− CD4+ splenocytes from WT or fyn−/− donors into Rag1−/− hosts. The CD45RBhighCD25− population consists of a TREG-depleted naive subset of CD4+ T cells; this CD4+ T cell fraction has been shown to undergo Th1-Th17 polarization when transferred into a lymphopenic host (45–49). We assessed Th1 and Th17 effector cytokine production from lymphocytes isolated from various organ compartments of recipient Rag1−/− mice 12 d postinjection (Fig. 5C). Analysis by intracellular staining and flow cytometry allowed the examination of cytokine production on a per-cell basis. In all compartments, viable cells from the fyn−/− donor produced less IL-17 compared with WT cells. However, fyn−/− cells produced comparable levels of IFN-γ, suggesting that the diminished IL-17 expression was not merely due to a general abrogation of inflammatory cytokine production. However, it should be noted that the fyn−/− CD45RBhighCD4+ T cells appeared to have a defect in homeostatic proliferation: we consistently recovered fewer fyn−/− cells from Rag1−/− hosts compared with WT cells (data not shown). Conceivably, the mechanism of IL-17 production may be tied to proliferation in a manner reminiscent of other cytokines, in which T cells undergo several rounds of division before becoming fully competent to express IL-4 or IFN-γ (50).

**Discussion**

Increasing evidence suggests that the TREG 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 TREG lineages by orchestrating the temporal expression or activation of STAT3, RORγt, and Foxp3. fyn−/− CD4+ T cells exhibited a profound delay in RORγt expression during the Th17-polarizing conditions did not fully upregulate the Th17-associated gene Il17. Instead, fyn−/− CD4+ T cells diverged into a TREG-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 expression 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). Though 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 increased 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 fy−/− Th17 cells during the late stages of differentiation are sufficient to drive the expression of IL21 and IL23r. In contrast, fy−/− 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 (59) 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 fy−/− 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 CD45RB+CD4+CD45R0+ T cells adoptively transferred into RagI−/− RagII−/− hosts also produced less IL-17 in the absence of Fyn (Fig. 5C), suggesting a T cell-intrinsic requirement for Fyn in the promotion of IL-17 expression by CD4+ T cells in vivo. Based on these data, we hypothesize that Fyn-deficient mice may be more resistant to Th17-mediated inflammation or autoimmune disease and that pharmacological inhibition of Fyn may be therapeutically beneficial in such disease settings.

Together, the results of this study suggest that Fyn is a mediator of Th17 differentiation and that it modulates the temporal activation and deactivation of STAT3, RORγt, and Foxp3. We also show that the deregulation of these transcription factors has differential effects on the expression of various Th17-associated genes. These findings underscore the fact that the precise regulation of myriad signaling pathways is necessary for efficient Th17 differentiation and suggest that Fyn plays a role in orchestrating this regulation.

Acknowledgments

We thank all past and present members of the Stein and Zhou Laboratories for helpful feedback and suggestions. Experimental support was provided by the Northwestern University Interdepartmental ImmunoBiology Flow Cytometry Core Facility, the Northwestern University Genomics Core, and the Northwestern Robert H. Lurie Comprehensive Cancer Center Flow Cytometry Facility and Cancer Center.

Disclosures

The authors have no financial conflicts of interest.

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