ImmunoHorizons

RESEARCH ARTICLE

Adaptive Immunity

STAT3 Signaling Heterogeneity Underlies Cytokine-Expressing Fate in Th17 Cultures

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ABSTRACT

The polarization of naive Th cells into differentiated subsets in vitro was a powerful approach to define the development and function of Th cells in vivo. Th cell cultures identified cytokines that promote polarization and defined the phenotype and stability of differentiated cells. One of the limitations of this approach is the heterogeneity of the differentiated culture, essentially with regard to what proportion of the culture is secreting the hallmark cytokine of interest. This heterogeneity has always been puzzling because all cells in the culture have been exposed to identical culture conditions. We examined this phenomenon using an *Il17f* lineage-tracing allele (*Cost*, Cre on seventeen transcript) crossed to stop-flox *Rosa-YFP* (yellow fluorescent protein) mice. We found that less than half of the cells in a Th17 culture become lineage-positive during a differentiation culture and that it is primarily cells that are lineage-positive that produce cytokines when cultures are restimulated after differentiation. We sorted and analyzed YFP-positive and YFP-negative cells and found similar expression of many Th17 transcription factors, although YFP-negative cells had increased expression of other lineage-defining transcription factors. We observed that YFP-negative cells had diminished expression of *Stat3* and *Il6ra*, as well as decreased STAT3 activation. YFP-negative cells transduced with active STAT3 had significant increases in IL-17A expression, without increases in Th17 transcription factors. Taken together, these data suggest that there is a threshold of STAT3 activation that is required for efficient Th17 differentiation, and that even in a culture of homogeneous naive T cells there is heterogeneity in the receipt of early cytokine signals. *ImmunoHorizons*, 2023, 7: 747–754.

INTRODUCTION

$CD4^+$ Th cells acquire varying functional properties that depend on the milieu of early cytokine exposure. Cytokines activate signaling factors that include STAT and Smad family members that promote differentiation and the expression of transcription factors that further reinforce a differentiated phenotype. Distinct subsets promote differentiation of Th subsets that are identified by expression of hallmark cytokines (1–4). Th subsets with polarized cytokine secretion patterns have been identified in various disease states (5–8), although there is some controversy over how readily they are identified in models of pathogen immunity (9).

Much of what we understand about Th subsets has been derived from the use of in vitro culture. Th cell subsets were first identified using T cell clones that developed from long-term culture (7, 10). Subsequent identification of cytokines that support polarized Th cell differentiation allowed the optimization of conditions for growth of large numbers of naive T cells into polarized Th subsets. Yet, a major limitation of these

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Abbreviations used in this article: Cost, Cre on seventeen transcript; gMFI, geometric mean fluorescence intensity; YFP, yellow fluorescent protein.

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cultures is that not all hallmark cytokines are expressed by all of the cells in the culture. There have generally been two largely hand-waving arguments about this observation. The first is that cytokine-negative cells are undifferentiated, even though all cells in culture have been exposed to the same polarizing cytokine environment. Another interpretation of this phenomenon is that intracellular cytokine staining only captures a window of cytokine production, and that seemingly cytokine-negative cells would be positive if they could be tracked at another time point. Neither of these hypotheses has been clearly tested.

In this study, we use *Il17f* lineage-tracing mice (11) to delineate the fate of cytokine-negative cells in Th17 cultures. In these mice, Cre expressed from the *Il17f* allele facilitates expression of yellow fluorescent protein (YFP) from the Rosa floxed/stop-YFP strain. During the course of differentiation, only about half of the cultured cells became lineage-positive, identifying cells that had ever expressed IL-17F during their time in culture. We found that YFP-negative cells had expression of lineage-defining transcription factors, albeit at slightly reduced levels, and an increase in T-bet expression. Strikingly, *Stat3* expression and STAT3 activation were decreased in YFP-negative cells, and transduction of an active STAT3 into YFP-negative cells resulted in increased IL-17 production without increases in other Th17-associated transcription factors. Taken together, these results suggest that cytokine-negative cells in a Th-polarized culture are differentiated but have early cytokine signaling that is suboptimal in generating a fully differentiated phenotype.

MATERIALS AND METHODS

Mice

Il17f$^{Cost/Cost}$ mice (11) used in in vitro experiments were between 6 and 8 wk of age. Experiments were performed in both male and female mice. All experiments were performed with the approval of the Indiana University Institutional Animal Care and Use Committee.

Cell lines

The Platinum-E cell line was a gift from Dr. Alexander Dent. Cells were cultured in DMEM containing 10% FBS (Atlanta Biologicals), 1% antibiotics (penicillin and streptomycin/stock: 5000 μg/ml penicillin, 5000 μg/ml streptomycin), 1 mM sodium pyruvate, 1 mM L-glutamine, 2.5 ml of nonessential amino acids (stock: 100×), 5 mM HEPES (all from Lonza), and 57.2 μM 2-ME (Sigma-Aldrich).

In vitro mouse T cell differentiation

CD4$^+$CD62L$^+$ cells were isolated from the spleens and lymph nodes of the mice using magnetic separation following the supplier's protocol (Miltenyi Biotec, Auburn, CA). Cells were cultured in RPMI 1640 media containing 10% FBS (Atlanta Biologicals), 1% antibiotics (penicillin and streptomycin/stock: 5000 μg/ml penicillin, 5000 μg/ml streptomycin), 1 mM sodium pyruvate, 1 mM L-glutamine, 2.5 ml of nonessential amino acids (stock: 100×), 5 mM HEPES (all from Lonza), and 57.2 μM 2-ME (Sigma-Aldrich). T cells were plated at a density of 300,000/well in a 48-well plate and activated with plate-bound anti-CD3 (2 μg/ml; Bio X Cell) and soluble anti-CD28 (2 μg/ml; Bio X Cell) Abs. Cells were differentiated to Th17 cells (100 ng/ml mouse IL-6, 10 ng/ml IL-1β, 10 ng/ml IL-23, 10 μg/ml anti–IL-4, and 10 μg/ml anti–IFN-γ), Th1 cells (10 ng/ml IL-12, 50 U/ml IL-2, and 10 μg/ml anti–IL-4), and Th2 (10 ng/ml IL-4, 10 μg/ml anti–IFN-γ, and 50 U/ml human IL-2), grown at 5% CO_2, and were expanded on day 3 with the original concentration of cytokines in fresh medium. Cells were collected on day 5 unless otherwise indicated for analysis.

Reverse transcription–quantitative PCR

RNA was harvested from large bulk populations or tissues at the indicated time points in TRIzol reagent (Life Technologies). cDNA was produced by reverse transcribing mRNA via a qScript cDNA synthesis kit (Quantabio). Real-time PCR was carried out with TaqMan primers (Life Technologies) using a 7500 Fast PCR machine (Life Technologies). Data were normalized to *B2m* expression.

Flow cytometry

For cytokine staining, cells were stimulated with PMA (100 ng/ml, Sigma-Aldrich) and ionomycin (100 ng/ml, Sigma-Aldrich) for 3 h followed by monensin (2 μM, BioLegend) for a total of 6 h at 37°C. After stimulation, cells were stained with a fixable viability dye (eBioscience) and Abs for surface markers for 30 min at 4°C, before fixation with 4% formaldehyde for 10 min in the dark at room temperature. After fixation, cells were permeabilized with permeabilization buffer (eBioscience) for 1 h at 4°C and stained for cytokines for 1 h at 4°C. For transcription factor staining, cells were stimulated with PMA and ionomycin (concentration as above) for 5 h. They were then stained with fixable viability dye and other surface markers for 30 min at 4°C before fixation with 1% paraformaldehyde for 10 min in the dark at room temperature. Cells were then incubated in Perm-Fix buffer (eBioscience) overnight. The next day, cells were permeabilized in permeabilization buffer for 1 h at 4°C and stained with transcription factors for 1 h at 4°C. After intracellular or transcription factor staining, cells were washed with FACS buffer and analyzed using the Attune NxT flow cytometer (Thermo Fisher Scientific) and with FlowJo 10.9 software.

Cell sorting

For cell sorting, cells were stimulated with PMA (100 ng/ml, Sigma-Aldrich) and ionomycin (100 ng/ml, Sigma-Aldrich) for 5 h in media. Cells were washed with FACS buffer twice and resuspended at concentration of 5 million/ml. Cells were sorted using a BD FACSAria fusion flow cytometer (BD Biosciences) and then processed for downstream analysis.

Retrovirus transfection

Platinum-E cells were grown to 80–90% confluency in 10 ml of DMEM with 10% FBS and 1% antibiotics in a 100-mm tissue culture dish. Cells were transfected with control vector or retroviral vector containing a STAT3c open reading frame by using Lipofectamine 3000 (Thermo Fisher Scientific). Eighteen micrograms of retroviral vector, 6 μg of pCL-Eco, and 50 μl of P3000 were mixed in 500 μl of Opti-MEM I reduced serum medium (Thermo Fisher Scientific), and 50 μl of Lipofectamine 3000 was mixed in another 500 μl of Opti-MEM I reduced serum medium. After combining, this mixture was incubated at room temperature for 15 min. The combined mixture was gently added to the Platinum-E cell culture dishes. After 16 h, the media containing the retrovirus were collected and replaced with fresh DMEM for 4 d. The media containing the virus was centrifuged at 1500 rpm for 5 min to remove cell debris. Virus supernatant was used for retroviral transduction or stored at −80°C for subsequent use.

Retrovirus transduction

Activated mouse CD4$^+$ T cells were plated in 48-well plates, the Th cell–conditioned media were removed, and STAT3c or control retrovirus supernatant in the presence of 8 μg/ml Polybrene (Sigma-Aldrich) was added to the plate. The plate was centrifuged at 2000 rpm at 25°C for 90 min without break. After spin infection, the supernatant was removed and the Th cell–conditioned media were added back to the plate. Cells were expanded on day 3 and analyzed on day 5.

Statistics and data analysis

All statistics were done using Prism software version 7 (GraphPad). A Student *t* test was used for the comparison of two samples. One-way ANOVA with a Tukey multiple comparison test was used for the comparison of three or more groups unless otherwise stated. Flow cytometry data were collected using an NxT Attune flow cytometer (Life Technologies) and were analyzed using FlowJo version 10.

RESULTS

Kinetics of Il17f *reporter allele expression in Th17 cultures*

To begin to characterize cytokine-secreting and nonsecreting populations in Th17 cultures, we took advantage of an *Il17f* lineage reporter allele that we previously generated (11). The allele was generated as a knockin/knockout of Cre to the *Il17f* gene that we termed the *Cost* (Cre on seventeen transcript) allele. Cost mice were crossed to *Rosa*-stop-flox YFP ($Rs^{+/YFP}$) and bred for homozygosity or heterozygosity at the *Cost* allele. Naive CD4 T cells were cultured under Th17 conditions and assessed daily for YFP expression. Cells from *Il17f* wild-type $Rs^{+/YFP}$ mice had <0.2% YFP-positive cells throughout differentiation

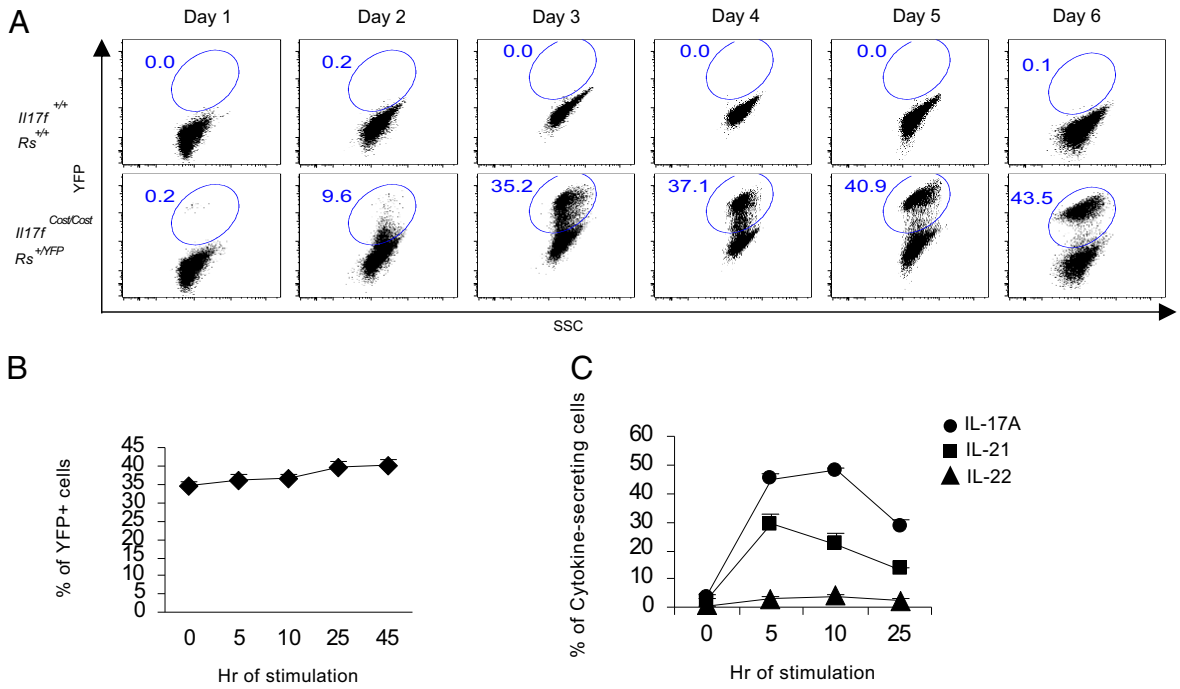

FIGURE 1. YFP and cytokine kinetics in *Il17f*$^{Cost/Cost}$ *Rs*$^{+/YFP}$ cells.

Splenic naive CD4$^+$ T cells from *Il17f*$^{Cost/Cost}$ *Rs*$^{+/YFP}$ mice were cultured in Th17 cell polarizing media. (**A**) Kinetics of YFP expression in *Il17f*$^{Cost/Cost}$ *Rs*$^{+/YFP}$ Th17 cells from days 1–6 of differentiation. (**B**) Kinetics of YFP expression in *Il17f*$^{Cost/Cost}$ *Rs*$^{+/YFP}$ Th17 cells after restimulation with PMA and ionomycin from 0 to 45 h. (**C**) Kinetics of IL-17A, IL-21, and IL-22 expression after restimulation with PMA and ionomycin from 0 to 25 h.

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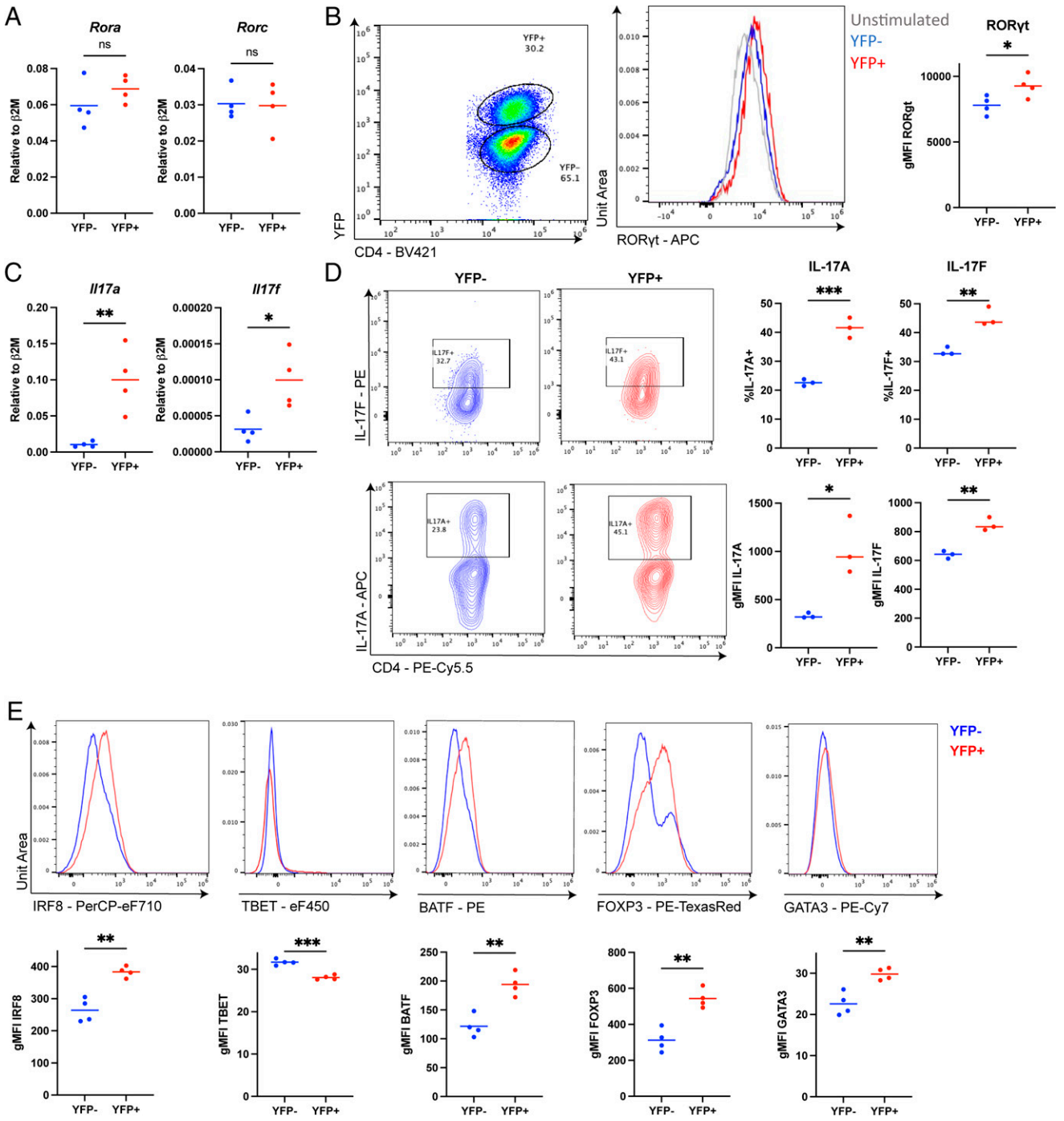

FIGURE 2. Cytokine and transcription factor characterization of YFP-negative and YFP-positive populations in *Il17f*$^{Cost/Cost}$ *Rs*$^{+/YFP}$ cells. Splenic naive CD4^{+} T cells from *Il17f*$^{Cost/Cost}$ *Rs*$^{+/YFP}$ mice cultured in Th17 cell polarizing media for 5 d. (**A**) mRNA quantification of *Rora* and *Rorc* by transcription–quantitative PCR (RT-qPCR) in sorted YFP-negative and YFP-positive cells. (**B**) RORγt expression in YFP-negative and YFP-positive cells assessed by flow cytometry. YFP-negative/YFP-positive gating (left), histogram of RORγt staining (middle), and gMFI RORγt quantification (right). (**C**) mRNA quantification of *Il17a* and *Il17f* by RT-qPCR in sorted YFP-negative and YFP-positive cells. (**D**) IL-17A and IL-17F expression in YFP-negative and YFP-positive cells assessed by flow cytometry. Representative flow plots (left) and quantification (right) are shown. (**E**) Flow cytometric staining of IRF8, TBET, BATF, Foxp3, and GATA 3 in sorted YFP-negative and YFP-positive cells. Representative histograms (top) and quantification (bottom) are shown. (**F**) mRNA quantification for *Stat3* and flow cytometric staining for total and p-STAT3. Quantification (left) and representative histograms (right) are shown. (**G**) mRNA quantification of *Il6ra*, *Il21*, and *Il21r* from sorted YFP-negative and YFP-positive cells. A Student *t* test was used for comparison of two groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

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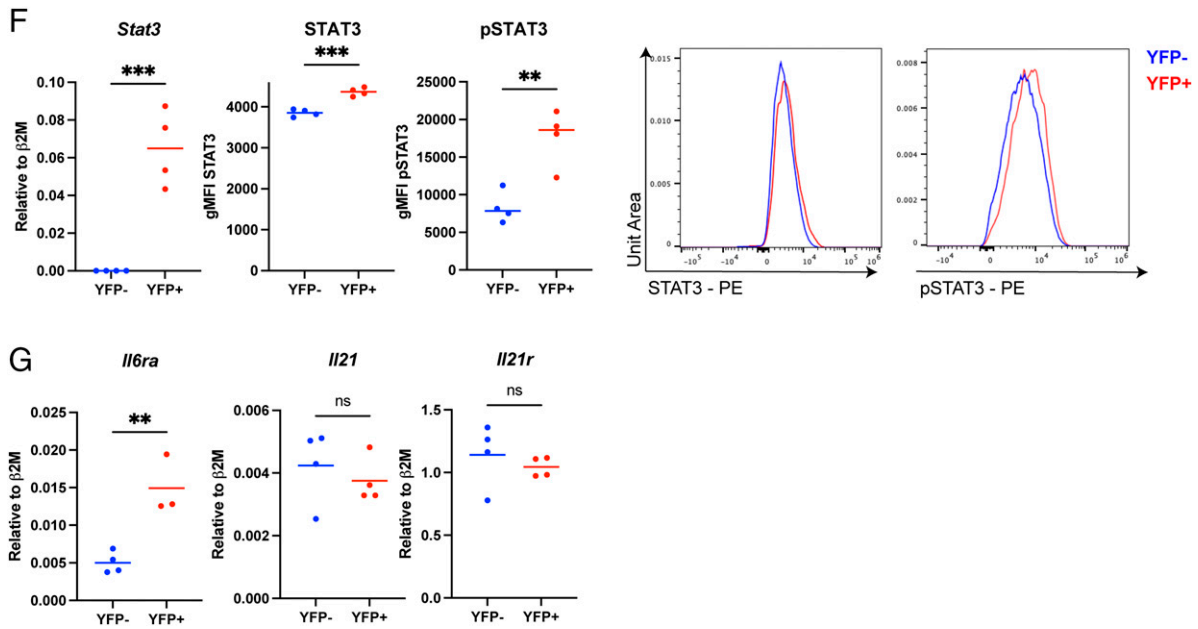

FIGURE 2. (*Continued*)

(Fig. 1A). In contrast, *Il17f*$^{Cost/Cost}$ *Rs*$^{+/YFP}$ cells acquired YFP-positive cells as early as day 1 of culture, and the percentage increased to 40–45% by the end of the 5-d culture (Fig. 1A). This suggested that less than half of the cells in culture had ever expressed Cre from the *Il17f* locus.

It was possible that YFP-negative cells were still primed to express the *Il17f* locus on restimulation. To test this, we took cells from 5-d cultures, restimulated them with PMA and ionomycin, and assessed YFP expression at various time points after stimulation. YFP-positive cells increased only modestly during 45 h of cultures (Fig. 1B), suggesting that most cells are not primed for rapid cytokine expression, and that it is the proportion of cells that already expressed IL-17F during culture that express cytokine upon restimulation. It is unlikely that there was a later peak of cytokine expression, as production of IL-17A and IL-21 peaked and waned during the first 24 h (Fig. 1C). These data suggest that in Th17 cultures, cells with or without cytokine-secreting potential are separable populations.

Transcription factor expression in cytokine-positive and -negative populations

To further distinguish the phenotype of YFP-positive and YFP-negative cells, we examined the expression of transcription factors that are hallmarks of Th lineages (Supplemental Fig. 1). *Rora* and *Rorc* were similar between the two populations at the mRNA level despite mRNA for *Il17a* being greater in YFP-positive cells (Fig. 2A, 2C). Intracellular staining for RORγt showed only modest but significant enrichment in YFP-positive cells, whereas there was a more dramatic enrichment in IL-17A–secreting cells in the YFP-positive cells (Fig. 2B, 2D). Intracellular staining for other transcription factors indicated enrichment of both IL-17–promoting and Th17-repressing factors in the YFP-positive population, although T-BET had greater expression in YFP-negative cells (Fig. 2E).

Surprisingly, we observed greatly diminished *Stat3* mRNA expression that correlated with significant differences in the mean fluorescence intensity (gMFI) of total STAT3 protein and p-STAT3 staining (Fig. 2F). This was consistent with lower *Il6ra* expression in YFP-negative cells, although there was no difference in expression of *Il21* or *Il21r* mRNA (Fig. 2G). Taken together, these data suggest that YFP-negative cells largely adopt a Th17 phenotype, albeit with lower expression of some Th17-associated transcription factors.

Active STAT3 induces IL-17 expression in YFP-negative cells

STAT3 is required for effective Th17 differentiation and can increase IL-17 production even when transduced into Th17 cultures (12, 13). Whereas STAT3 induces the expression of several IL-17–promoting transcription factors, it also directly interacts with the *Il17a–Il17f* loci to counteract the repressive effects of STAT5 (14, 15). To determine whether increasing STAT3 signaling would increase IL-17A production from YFP-negative cells, we transduced YFP-negative Th17 cells with a control vector or a constitutively active STAT3 retrovirus and gated on retrovirus reporter-positive cells for analysis (12, 16). The change in p-STAT3 gMFI between empty vector–transduced YFP-negative cells and *Stat3*c-transduced cells was similar to the change in gMFI between YFP-negative and YFP-positive cells, indicating restoration of STAT3 signaling in YFP-negative cells to a level similar to that in YFP-positive cells (data not shown). We observed that active STAT3-transduced cells had an increased frequency of YFP-positive cells (Fig. 3A). This was associated with increased IL-17A production (Fig. 3B) without increasing

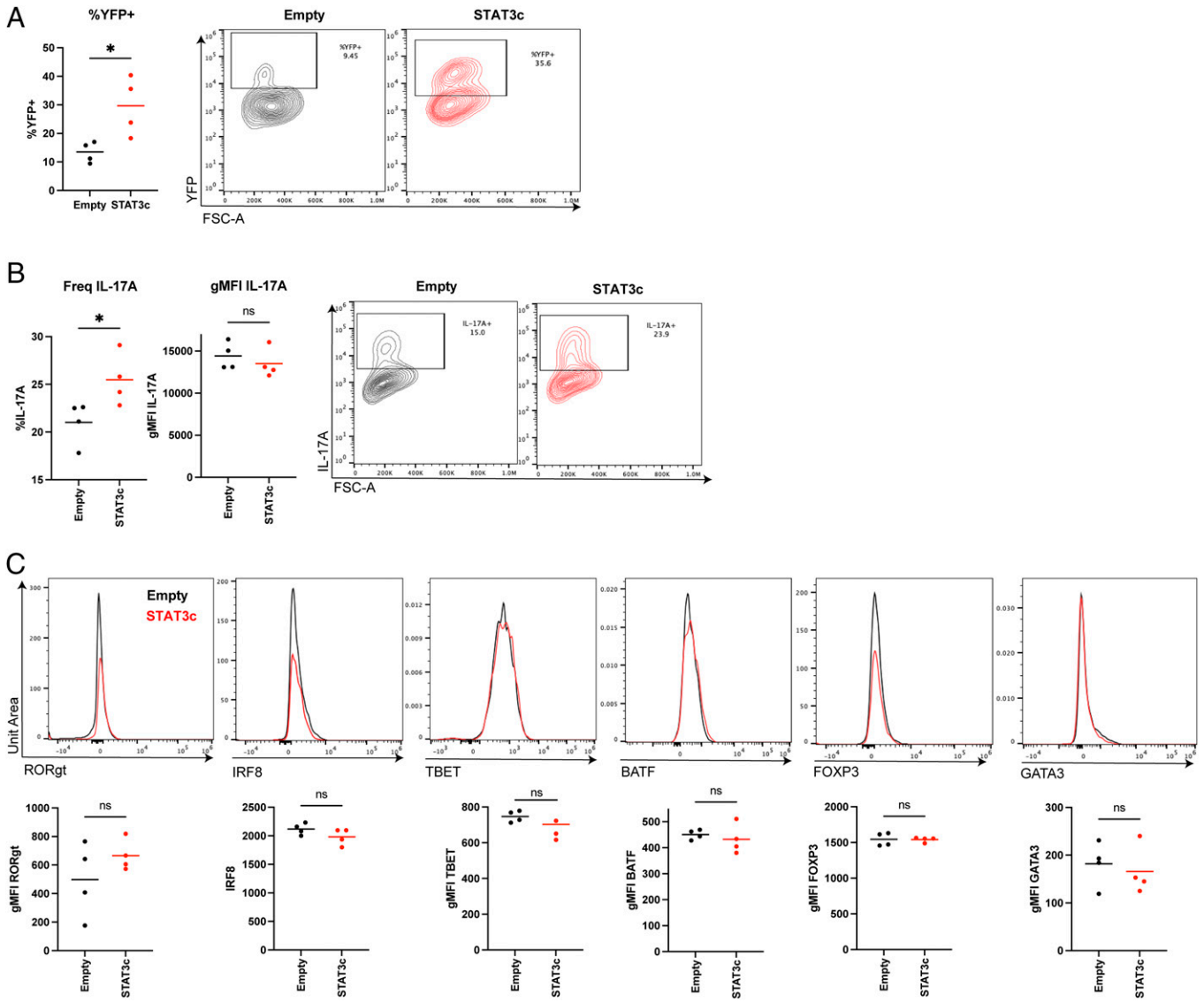

FIGURE 3. STAT3 enhances IL-17A expression in YFP-negative cells without altering transcription factor levels.
Splenic naive CD4$^+$ T cells from *Il17f*$^{Cost/Cost}$ *Rs*$^{+/YFP}$ mice were cultured in Th17 cell polarizing media for 5 d and then sorted for YFP-negative cells. YFP-negative cells were transduced with either an empty construct or construct containing a constitutive active STAT3 open reading frame. H-2K^k encoded on both constructs was used to select for transduced cells. (**A**) Frequency of YFP-positive cells induced by either the empty vector or the STAT3c vector transduction into YFP-negative cells. Quantification (left) and representative flow plots (right) are shown. (**B**) IL-17A expression induced by transduction of empty or STAT3c-containing vector into sorted YFP-negative cells. Populations were gated on retrovirus reporter-positive cells. Quantification (left) and representative flow plots (right) are shown. (**C**) Flow cytometric staining of RORγt, IRF8, TBET, BATF, Foxp3, and GATA3 in YFP-negative cells transduced with the empty or STAT3c vector. Representative histograms (top) and quantification (bottom) are shown. A Student *t* test was used for comparison of two groups. *$p < 0.05$.

expression of other Th17-associated transcription factors (Fig. 3C). These data suggest that STAT3 signaling is one limiting factor in Th17 cultures and that cytokine-negative cells likely experienced less STAT3 activation during the differentiation process.

DISCUSSION

Heterogeneity in Th cell cultures was recognized early in the development of the field. Even in optimized cultures, all cells at the end of the culture period did not stain for cytokine, despite the starting material being purified naive T cells exposed to similar culture conditions. Although various explanations for this phenomenon have been proposed, it has not been carefully investigated. In this study, we used an IL-17F lineage tracer allele and found that Th17 cells that were cytokine-negative or -positive after 5 d of differentiation were separable populations. Both had features of Th17 cells including similar expression of *Rorc* and *Rora*, hallmarks of Th17 cells, but YFP-negative cells

had greatly diminished IL-17A production. Moreover, there was not an increase in YFP-positive cells following restimulation, suggesting that cells that had become YFP-positive during differentiation were the same cells producing cytokine on restimulation. YFP-negative cells did display signs of diminished STAT3 activity, and ectopic expression of active STAT3 resulted in greater IL-17A production in YFP-negative cells. Expression of active STAT3 also increased YFP expression in previously YFP-negative cells. This phenotype is likely due to direct activation at the *Il17f* locus, but it is also possible that transduction of active STAT3 may confer progrowth and survival effects on the Th17 cells that may select for this phenotype.

The identification of STAT3 as a factor does not eliminate the potential role of other cytokine signaling receptors and factors in control of these subsets. The use of constitutively active STAT3c was designed to overcome any differences in upstream signaling factors and receptors. *Il6ra* levels were decreased in YFP-negative cells compared with YFP-positive cells, and IL-6 was used to stimulate Th17 cells for assessment of STAT3 expression. By using constitutively active STAT3, we bypassed any effects due to differential expression of *Il6ra* between YFP-negative and YFP-positive cells. However, whether the diminished expression of *Il6ra* in the YFP-negative cells contributes to diminished IL-17 production remains to be investigated. Additionally, IL-2 inhibits Th17 cell differentiation and IL-17 expression by activating STAT5 (15). It would be interesting to further compare IL-2 responsiveness in YFP-negative and YFP-positive cells and to determine whether varying levels of IL-2 signal may also be linked to differentiation of these subpopulations.

We previously examined heterogeneity in Th2 cells, based on the observations that not only do not all cells express cytokine, but in examining multiple Th2 cytokines, we observed that not all cells expressing one cytokine necessarily expressed others. We observed that segregated expression of PU.1 in IL-4–negative cells and gradients of IRF4 expression accounted for at least some of the heterogeneity in Th2 cultures (17, 18). We observed additional heterogeneity in cultures caused by gradients of PU.1-regulated TCR expression in primary T cells (19). These results suggest that heterogeneity in transcription factor expression underlies heterogeneous Th2 cytokine expression.

There is already recognized heterogeneity among Th17 populations that depends on the cytokines used to polarize cultures (20, 21). Studies indicated that Th17 cells cultured with TGF-β were less inflammatory than Th17 cells cultured with IL-1β in combination with IL-6 and/or IL-23 (20, 21). Th17 cells derived from TGF-β stimulation had higher IL-17 than did IL-1β+IL-23–derived cells, but also greater production of IL-10 that altered their function. Later studies demonstrated the unique role of IL-23 in the priming function of memory Th17 cells (22) Th17 cells generated with or without TGF-β had transcription factor profiles consistent with Th17 cells. Yet, despite similar transcription factor profiles, these populations had distinct functions in vivo.

Results from the current study extend the concept of transcription factor–driven heterogeneity in Th17 cells. In addition to variation in the expression of lineage-associated and opposing lineage-defining transcription factors, we also observed differences in the cytokine signals that promote Th17 differentiation. Expression of *Il6ra* and *Stat3* were significantly lower in the YFP-negative Th17 cells. This suggests that heterogeneity in cytokine signaling, due either to variation in receptor or signaling protein expression, results in differences in outcomes even in the context of in vitro differentiation. It further suggests that a threshold exists for cytokine signaling to activate a cytokine locus during differentiation and that when that threshold is not achieved, cells can still acquire a Th17 transcription factor profile but not the ability to activate the *Il17a/f* loci. It would be interesting to investigate such a threshold in other Th cell subsets that demonstrate a similar heterogeneity such as previously described Th2 cell heterogeneity (17, 18).

This study also raises the philosophical question of whether Th17 cells can be termed a Th17 cells when they have expression of key Th17 cell transcription factors but do not make IL-17. We would propose that the transcription factor profile and likely the chromatin structure identify these cells as Th17 and recognize that even within the Th17 phenotype, there is diversity. It is possible that Th17 cells (defined by transcription factor expression or chromatin structure) that do not express IL-17 might have distinct functions that are yet to be defined.

DISCLOSURES

The authors have no financial conflicts of interest.

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