HHS Public Access

Author manuscript

J Immunol. Author manuscript; available in PMC 2022 September 01.

Published in final edited form as:

J Immunol. 2021 September 01; 207(5): 1265–1274. doi:10.4049/jimmunol.2100165.

STAT5 represses a STAT3-independent Th17-like program during Th9 cell differentiation

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Abstract

IL-9-producing T helper cells, termed Th9 cells, contribute to immunity against parasites and cancers but have detrimental roles in allergic disease and colitis. Th9 cells differentiate in response to IL-4 and TGF-β, but these signals are insufficient to drive Th9 differentiation in the absence of IL-2. IL-2-induced STAT5 activation is required for chromatin accessibility within *Il9* enhancer and promoter regions and directly transactivates the *Il9* locus. STAT5 also suppresses gene expression during Th9 cell development, but these roles are less well defined. Here we demonstrate that human allergy-associated Th9 cells exhibited a signature of STAT5-mediated gene repression that is associated with the silencing of a Th17-like transcriptional signature. In murine Th9 cell differentiation, blockade of IL-2/STAT5 signaling induced the expression of IL-17 and the Th17-associated transcription factors Rorγt. However, IL-2-deprived Th9 cells did not exhibit a significant Th17- or STAT3-associated transcriptional signature. Consistent with these observations, differentiation of IL-17-producing cells under these conditions was STAT3-independent, but did require Rorγt and BATF. Further, ectopic expression of Rorγt and BATF partially rescued IL-17 production in STAT3-deficient Th17 cells, highlighting the importance of these factors in this process. While STAT3 was not required for the differentiation of IL-17-producing cells under IL-2-deprived Th9 conditions, their prolonged survival was STAT3-dependent, potentially explaining why STAT3-independent IL-17 production is not commonly observed in vivo. Together, our data suggest that IL-2/STAT5 signaling plays an important role in controlling the balance of a Th9 vs a Th17-like differentiation program in vitro and in allergic disease.

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Introduction

CD4$^+$ T helper (Th) cells differentiate in response to inflammatory cytokines and downstream signaling through receptor-associated signal transducer and activator of transcription (STAT) factors. For example, interleukin (IL)-4-producing Th2 cells differentiate in response to IL-4 and signaling through STAT6 (1). However, in the presence of transforming growth factor (TGF)-β, IL-4-driven Th2 cell development is subverted and results in the differentiation of IL-9-producing Th9 cells that play detrimental roles in allergic disease and exhibit enhanced antitumor immunity after adoptive cell therapy (2–4). During the differentiation process, IL-4-induced STAT6 binds regulatory elements within the *Il9* locus and additionally suppresses expression of transcription factors (i.e. T-bet, Foxp3) that inhibit Th9 differentiation (2, 5). Similarly, TGF-β induces expression of PU.1 which diverts cells from a Th2 to a Th9 phenotype (6). Despite the importance of these cytokines and transcription factors in Th9 differentiation, they are insufficient to drive Th9 differentiation in the absence of IL-2/STAT5 signaling.

IL-2 is a pleiotropic cytokine that acts to drive the proliferation, survival and differentiation of Th cells by binding the IL-2 receptor complex and inducing the phosphorylation of STAT5 (7). Upon phosphorylation, STAT5 forms homodimer or tetrameric structures that translocate to the nucleus where they either activate or suppress gene expression based on the molecular context of each bound loci. While IL-2/STAT5 signaling is detrimental for the differentiation of Th17 and T follicular helper cells, it enhances Th1 and Th2 lineage programs (8–11). In previous work, we and others have shown that IL-2 and STAT5 signaling also promote the differentiation and cytokine producing capacity of Th9 cells (12–15). In the differentiation process, STAT5 represses expression of BCL6 which competes for binding with STAT5 at the *Il9* locus and likely other Th9-associated gene loci (12, 13). Additionally, STAT5 plays a “pioneering” role in Th9 cell differentiation by facilitating the binding of other transcription factors (TFs) (i.e. BATF and IRF4) to the *Il9* locus (16–18). IL-2 and STAT5 also enhance the cytokine producing capacity of Th9 cells post-differentiation. In this role, IL-2-production by Th9 cells feeds back in a paracrine fashion and amplifies production of IL-9 and several other STAT5-induced cytokines after T cell receptor activation (14). Despite the known roles on IL-2 and STAT5 signaling in the in vitro differentiation and function of Th9 cells, much less is known about how these signals impact the differentiation of Th9 cells during disease. Further, how IL-2/STAT5 signaling impacts Th9 differentiation outside of repressing BCL6 and modulating the *Il9* locus has not been well established.

We demonstrate here that STAT5 signaling is associated with IL-9-producing Th cells in human allergic disease. While a number of known STAT5 target genes were induced in human Th9 cells, we also observed a strong signature of STAT5-mediated gene repression in IL-9-producing cells. STAT5-mediated gene repression correlated with the suppression of a Th17-like signature, indicating that STAT5 may regulate the balance of Th9/Th17 differentiation during disease. In a murine Th cell differentiation model, we further demonstrated that IL-2-deprived Th9 cells produced high levels of IL-17 and the Th17-associated factors Rorγt and BATF. Despite their capacity to produce IL-17, IL-2-deprived Th9 cells did not exhibit a classical Th17 cell transcriptional profile and

surprisingly maintained IL-17 production and Rorγt and BATF expression in the absence of STAT3. STAT3-independent IL-17 production, however, was dependent on both Rorγt and BATF. Despite the potential for IL-17-producing cells to differentiate independently of STAT3 in these conditions, STAT3 was required for the prolonged survival of IL-2-deprived Th9 cells. These data may explain why STAT3-independent IL-17-producing Th cells are not readily apparent in prolonged in vivo models of disease. Together, our data identify an important role of STAT5-mediated gene repression in human and mouse Th9 cell development and define a novel STAT3-independent pathway involved in the differentiation of IL-17-producing T cells. Insight into this STAT3-independent pathway may be useful for overcoming STAT3 loss of function immunodeficiencies that feature a lack of IL-17-producing Th cells and susceptibility to multiple opportunistic pathogens.

Material and Methods

Mice

C57BL/6 mice were bred in-house at Purdue University. *Stat3* $^{fl/fl}$ mice expressing *Cd4-Cre* recombinase, *Stat3* $^{fl/fl}$ *Cd4*^{cre}, were originally obtained from Dr. D. Levy (New York University, New York, NY). *Batf* $^{-/-}$ mice were kindly provided by Dr. Elizabeth Taparowsky (Purdue University, West Lafayette, IN). All mice experiments were in compliance with the protocol approved by the Purdue Animal Care and Use Committee (PACUC).

In vitro T cell culture

Naïve CD4^{+} T cells were obtained from spleens and lymph nodes using the Naïve CD4^{+} T cell Isolation Kit (Miltenyi Biotec, Auburn, CA). Purity was >90% checked through flow cytometry. 1×10^{6} enriched cells/mL were cultured in a 1:1 mixture of RPMI or DMEM medium (Gibco) supplemented with penicillin-streptomycin (Gibco), 10% Fetal Bovine Serum and 50μM 2-mercaptoethanol (Gibco). Cells were cultured in αCD3ε (2μg/mL, 2C11, BioXcell) coated-plates and soluble αCD28 (4μg/mL, 37.51, BioXcell) under the following T cell polarization conditions: Th9 conditions- recombinant murine IL-4 (10 ng/mL, Peprotech), αIFNγ (12.5 μg/mL, XMG1.2, BioXcell), recombinant human TGF-β1 (4ng/mL, Peprotech), and αIL10R (12.5 μg/mL, 1B1.3A, BioXcell); Th17 conditions- αIL-4 (12.5 μg/mL, 11B11, BioXCell), αIFNγ, recombinant murine IL-6 (100ng/mL, Peprotech), TGF-β1 and αIL-10R. Additionally, in some conditions we neutralized IL-2 in Th9 and Th17 cultures with αIL-2 (5μg/mL, S4B6–1, BioXcell) and αCD25 (10μg/mL, 3C7, Biolegend). The cells were incubated at 37°C and harvested on day 4 of culture. In some experiments, either the RORγt specific inhibitors Digoxin (10μM, Cayman Chemical) or TMP778 (2.5μM, Aobious) or the corresponding dilution of DMSO (Fisher Bioreagents) were added to the conditions mentioned above. In long-term cell culture experiments, cells from each condition were harvested at day 4 and rested for 24 hours in the absence of anti-CD3 with ½ concentrations of the original cytokines. After this rest period, cells were re-seeded at 10^{6} cells/ml into fresh anti-CD3 coated wells and cultured for an additional 4 days under the initial conditions stated at which time cells were assessed for viability and expression of Th lineage-specific cytokines and transcription factors.

Intracellular staining and flow cytometry

At day 4 of culture, cells were harvested and 2×10^5 cells at 10^6 cells/mL were used for either cytokine staining (ICS) or transcription factor (TF) staining. For ICS, the differentiated Th cells were incubated at 37°C with PMA (0.5μg/mL) and ionomycin (0.5μg/mL, Sigma) for 2.5 hours and monensin (2μM) (Biolegend) was added for additional 2.5 hours. The cells were subsequently stained with fixable viability dye (eFlour 780, Thermo Fisher Scientific) and mouse anti-CD4 antibody, (RM4–5, 1μg/ml, Biolegend) and then were fixed with 3.7% formaldehyde. After fixation, cells were permeabilized with Intracellular Staining Perm Wash Buffer (Biolegend) and stained in the same buffer with a cytokine antibody cocktail including anti-IL-17A (TC11–18H10.1), anti-IL-9 (RM9A4), anti-IL-2 (JES6–5H4), anti-GM-CSF (MP1–22E9) and anti-TNF (MP6-XT22) (all purchased from Biolegend and used at 1μg/ml). For TF staining, the cells were fixed instead with True-nuclear™ fixation buffer (Biolegend). After fixation, the cells were permeabilized with True-nuclear™ Perm buffer (Biolegend), and were stained with antibodies to BATF (S39–1060, 1:100 dilution of stock, BD Biosciences), Rorγt (Q31–378, 2μg/ml, BD Biosciences) and IRF4 (IRF4.3E4, 0.5μg/ml, Biolegend). Stained samples were run on an Attune NxT flow cytometer (Thermo Fisher scientific), and the data were analyzed using FlowJo software (v.10.0).

Retroviral transduction

Retroviral particles were generated using the bicistronic vectors MSCV-IRES-eGFP and MSCV-Thy1.1 for RORγt and BATF (gifts from Dr. Mark Kaplan, Indiana School of Medicine and Dr. Elizabeth Taparowsky, Purdue University, respectively). Plasmids (10μg) containing each gene of interest were co-transfected with 5μg of packaging vector pCL-Eco (a gift from Dr. Mark Kaplan, Indiana School of Medicine) into HEK-293T cells using Lipofectamine 2000 according to manufacturer's instructions. At 48 hours post-transfection virus containing cell supernatant was collected and used for Th cell transduction. Briefly, naïve CD4 T cells were cultured under Th17 conditions as per above and on day 2 of culture, cells were "spinfected" at 2,000 rpm for 1.5 hours at 37°C in the presence of polybrene (8μg/mL, Sigma). Cells were harvested 2 days after transduction and the efficiency of the transduction as well as intracellular IL-17 production was assessed via flow cytometry.

Data analysis and Statistics

Data plots and statistics were generated using the software GraphPad Prism, V8. Statistics tests used are indicated in the figure legends and were considered significant if $p < 0.05$.

Bulk RNA-seq data analysis

The fastq files were downloaded from GSE41317. RNA expression levels in each library were estimated by "rsem-calculate-expression" from RSEM v1.3.0 (19) using default parameters except "--bowtie-n 1 –bowtie-m 100 –seed-length 28". The bowtie index (GRCm38) required by RSEM software was generated by "rsem-prepare-reference" on all RefSeq genes, obtained from UCSC table browser on April 2017. EdgeR v3.24.3 (20) package was used to identify differentially expressed genes between IL2 knock-out and

wildtype samples. Gene set enrichment analysis (GSEA) (21) was performed by GSEA version 3.0 using default settings except “permutation type = gene_set” and “Collapse = No_Collapse”.

Single cell RNA-seq analysis

The raw single-cell RNA-sequencing data from allergen-specific T cells in allergy and asthma patients were sourced from GSE146170 (22). All data were re-analyzed according to what has been described in the original manuscript. Briefly, cellranger count (v3.1.0) was used to align sequencing reads to GRCh38 reference genome and to extract barcodes and UMI counts.

Seurat v3 standard integration workflow was followed to enable comparative analysis among samples (23). Genes expressed in < 3 cells, cells with < 200 expressed genes and/or > 5% mitochondrial genes were removed from downstream analyses in each dataset. The filtered count matrices were then normalized by total UMI counts, multiplied by 10,000 and transformed to natural log space. The top 2000 variable features were determined based on the variance stabilizing transformation function (FindVariableFeatures) by Seurat with default parameters. All samples were integrated using canonical correlation analysis (CCA) function with default parameters. Potential artifacts arising from library size and percentage of mitochondrial genes were regressed out by the ScaleData function. To perform clustering, principal component analysis (PCA) was first performed and the top 50 Principal components (PCs) were included in a uniform manifold approximation and projection (UMAP) dimensionality reduction. Clusters were then identified on a shared nearest neighbor (SNN) modularity graph using the top 50 PCs and the original Louvain algorithm. Cluster annotations were derived from the same marker genes used in the original paper (22). Seurat function AddModuleScore (24) was used to calculate gene list (module) scores. This function calculates the average scaled expression levels of each gene list, subtracted by the expression of control sets. The expression of individual genes across selected cell types were extracted, plotted by GraphPad Prism V8, and compared using wilcoxon-test. Additional statistical tests used are indicated in the figure legends and were considered significant if $p < 0.05$.

Results

STAT5-mediated gene repression is a hallmark of human Th9 cells during allergic responses.

IL-2-mediated STAT5 signaling plays a critical role in the in vitro differentiation of Th9 cells by acting as both a transcriptional activator and repressor (12, 13, 15, 16). However, less is known about the roles of IL-2/STAT5 signaling in human Th9 cell development in vivo. To address this, we reanalyzed recent single cell transcriptomics data from house dust mite (HDM) allergic patients where *IL9*–producing (*IL9*^{hi}) and low *IL9*-producing (*IL9*^{low}) “Th2” cells populations were readily apparent (Fig 1A, Supp Fig 1A) (22). Consistent with the original study, both *IL9*^{hi} and *IL9*^{low} cells were enriched in HDM allergic, but not in asthmatic patients with no HDM sensitivity, as compared to healthy individuals. Further these subsets of cells were distinct from other Th lineages (i.e. Th1, type I IFNR^{+} cells

(ThIFNR) or activated Th cells (ThACT)) within these patients whose presence was less affected by disease status (Supp Fig 1 A-E). Collectively, these data suggest that *IL9*^{hi} and *IL9*^{low} Th cells are associated with allergic disease.

Based on these data, we reasoned that a direct comparison of *IL9*^{hi} and *IL9*^{lo} cells may help identify how STAT5 signaling shapes pathogenic Th cells during the allergic response. To address this point, we initially examined the expression of a number of known STAT5-regulated genes in *IL9*^{hi} and *IL9*^{lo} cells. While the levels of *IL4* were similar in *IL9*^{hi} and *IL9*^{lo} subsets (Fig 1A), other STAT5 target genes such as *IL5*, *IL13, IL1RL1,* and *GATA3* were more highly expressed in *IL9*^{hi} versus *IL9*^{lo} Th cells (Fig 1A, Supp Fig 1D-E). To test whether other STAT5 target genes were differentially affected, we sourced a list of IL-2-induced or repressed genes in Th9 cells (13) with known STAT5 binding (25) as a surrogate for direct in vivo induced and repressed targets of STAT5 (see Supp Table 1). Expression of STAT5-induced genes were only moderately higher in *IL9*^{hi} vs *IL9*^{lo} Th cells in HDM allergic patients (Fig 1B). Surprisingly, however, the expression of STAT5-repressed genes were markedly lower in *IL9*^{hi} vs *IL9*^{lo} cells (Fig 1C), suggesting that STAT5-mediated gene repression is enhanced in IL-9-producing cells and thus may have a role in their differentiation.

STAT5 has previously been shown to inhibit Th17 cell differentiation (8), thus we speculated that *IL9*^{lo} cells might exhibit a Th17-like signature as compared to *IL9*^{hi} cells. To test this, we sourced a list of Th17 signature genes (26) (see Supp Table 1) and compared the expression of these genes in *IL9*^{lo} vs *IL9*^{hi} cells. Although the expression of *IL17* and *RORC* were below the level of detection within these single cell data, *IL9*^{lo} cells did exhibited higher expression of Th17-associated genes as compared to *IL9*^{hi} cells (Fig 1D). Together, these data indicate that human Th9 cell differentiation is associated with enhanced STAT5 activity and that STAT5 potentially represses a Th17-like phenotype in IL-9-producing cells.

IL-2 signaling suppresses a Th17-like program in developing Th9 cells

As we observed a strong STAT5-mediated gene repression signature that was inversely correlated with a Th17-like signature in *IL9*^{lo} cells, we questioned if STAT5 signaling repressed a Th17-like differentiation program during Th9 cell development. To this point, we utilized a well-characterized in vitro murine model of Th9 cell differentiation where naïve CD4 T cells were cultured under standard Th9-polarizing conditions in the presence or absence of IL-2- and CD25-blocking antibodies. After 4 days of culture, we examined the expression of IL-9 and several Th17-associated proteins (i.e. IL-17A, IL-17F, Rorγt, IRF4, BATF) by flow cytometry. As previously reported, inhibition of STAT5 signaling through IL-2 blockade resulted in an almost complete loss of IL-9 production by Th9 cells and a reduction in the expression of the STAT5 target IRF4 (Fig 2A-C) (13, 15, 27). As predicted by our analysis of human *IL9*^{hi} and *IL9*^{lo} cells, we observed a striking increase in the protein expression of IL-17A, IL-17F and Rorγt that was similar or equivalent to that of bona fide Th17 cells cultured with anti-IL2/CD25 antibodies (Fig 2A-C). To further confirm these findings, we reanalyzed existing RNA-seq data (13), comparing WT and IL-2-deficient Th9 cells. IL-2-deficient Th9 cells exhibited dramatic reductions in the STAT5-induced genes

Il9, *Irf4* and *Batf3* as originally described (13). Similar to our protein data, we observed a dramatic induction of Th17-associated genes *Il17a*, *IL17f*, and *Rorc* as well as *Maf* and *Ccr6* in the absence of IL-2 in these Th9 cells (Fig 2D). Together, these data indicate that IL-2/STAT5 suppresses a Th17-like phenotype in Th9 cells and defines a transcriptional signature of Th9-derived IL-17-producing cells.

To examine the functional features of IL-2-deprived Th9 cells, we compared the transcriptomes of IL-2-deficient (KO) and sufficient (WT) Th9 cells against Th17-related programs using gene set enrichment analysis (GSEA). Despite the increased expression of *Il17* and *Rorc* in IL-2-deficient cells, there was not a significant correlation with the transcriptomes of these cells and that of the core Th17 signature (Fig 2E). However, genes that were more highly expressed in IL-2-sufficient WT cells, as compared to IL-2-deficient Th9 cells, were enriched in genes that antagonize Th17 cell differentiation (Fig 2E). Further, IL-2-deprived Th9 cells had an enhanced capacity to produce the neuroinflammatory cytokine GM-CSF, IL-2 and a reduced capacity to produce IL-17F as compared to bona fide Th17 cells (Fig 2F). These data indicate that while IL-2-deprived Th9 and Th17 cells share a number of similarities, they differ in their capacity to produce pro-inflammatory cytokines and likely require unique cytokine signaling and transcription factor networks for their differentiation.

STAT3-independent production of IL-17 in IL-2-deprived Th9 cell cultures

As IL-2-deprived Th9 cells did not resemble canonical Th17 cells at the transcriptional level, we questioned if these cells also differed in other key aspects of the Th17 differentiation pathway. Differentiation of Th17 cells requires IL-6- and IL-23-induced STAT3 signaling (8, 28–31). However, IL-2-deprived Th9 cells, as cultured here, lack exogenous STAT3 activating cytokines (i.e. IL-6, IL-10 and IL-21, (27)) and did not exhibit a transcriptomic similarities with genes induced by STAT3, but instead were modestly correlated with STAT3-suppressed genes (Fig 3A). Based on these data, we hypothesized that IL-17-producing IL-2-deprived Th9 cells may develop in a STAT3-independent fashion. To test this hypothesis, we isolated naïve CD4 T cells from *Stat3*$^{fl/fl}$ *Cd4*-Cre^{-} (i.e. WT STAT3 expression) or *Stat3*$^{fl/fl}$ *Cd4*-Cre^{+} (i.e. T cell-specific deletion of STAT3) and cultured these cells under Th9 or Th17 conditions in the presence or absence of IL-2 and CD25 blocking antibodies. In agreement with previous studies (31), IL-17A and IL-17F production in canonical Th17 conditions was completely dependent on STAT3 (Fig 3B, C). However, in IL-2-deprived Th9 polarizing conditions, production of IL-17A and 17F were STAT3-independent (Fig 3B, C), indicating that STAT3 is not required for IL-17 production under these conditions.

In Th17 cells, STAT3 is also required for the optimal expression of the Th17 master transcription factor Rorγt and the “accessory” transcription factors BATF and IRF4. As STAT3 was not required for IL-17 production under IL-2-deprived Th9 cell polarizing conditions, we questioned if expression of Rorγt and BATF were also STAT3-independent. Similar to IL-17, we observed virtually identical levels of Rorγt, BATF and IRF4 in WT and STAT3-deficient IL-2-deprived Th9 cells, whereas all of these factors were decreased in STAT3-deficient Th17 cells (Fig 3D, E). Taken together, these data indicate that STAT3 is

dispensable for induction of IL-17 and several Th17-associated transcription factors under IL-2-deprived Th9 polarizing conditions.

Prolonged culture of Th9 cells results in STAT3-independent IL-17 production

In our previous work we showed that the prolonged culture of Th9 cells resulted in reduced IL-2 bioavailability and the subsequent emergence of cells with the capacity to produce IL-17 (32). Moreover, increased IL-17 production in these settings correlated with enhanced levels of activated STAT3. Despite this appealing correlation, it was unclear whether or not STAT3 was required for IL-17 production in this setting. To address this, we repeated our initial prolonged culture experiments (32) with both WT and STAT3-deficient naïve CD4 T cells. Similar to our previous report, prolonged culture of WT Th9 cells resulted in a reduced frequency of IL-9-producing cells and an increase proportion of IL-17-producing cells as compared to day 4 of culture (Fig 4A, Fig 2A). Likewise, STAT3-deficient cells exhibited a similar capacity to produce IL-17 and had Rorγt and BATF expression levels that were not significantly different ($p>0.05$) than WT Th9 cells at this time point (Fig 4A-C). These data support our initial findings and indicate that IL-2/STAT5 signaling suppresses a STAT3-independent Th17-like phenotype in differentiating Th9 cells.

STAT3 is required for the prolonged survival of IL-2-deprived Th9 cells

Previous work has shown that STAT3 is required for the generation of IL-17-producing Th cells in vivo (33, 34). However, this lack of IL-17 production is potentially complicated by the poor survival and persistence of STAT3-deficient Th cells in these settings (30, 33). To test whether or not STAT3 is required for the persistence of IL-2-deprived Th9 cells, we utilized a prolonged culture approach where naive WT or STAT3-deficient CD4 T cells were cultured under Th9 polarizing conditions with and without IL-2 blockade for two rounds of culture as indicated in Fig 4. After the second round of culture, live cell counts and the frequency of viable cells (Supp Fig 2A) from each condition were assessed by flow cytometry. Under standard Th9 conditions, there was little difference in the total number or frequency of viable cells after 2 rounds of culture. However, when Th9 cells were deprived of IL-2, STAT3-deficient cells exhibited significantly ($p<0.05$) poorer survival as compared to their WT counterparts (Supp Fig 2B). These data indicate that STAT3 is required for the prolonged survival of IL-17 producing cells when IL-2 is limiting.

Rorγt is required for STAT3-independent IL-17 production

In Th17 cells, Rorγt directly binds the *Il17* locus and is required for IL-17 expression (28). Above, we demonstrated that IL-2-deprived Th9 cells expressed Rorγt in a STAT3-independent manner, indicating that IL-17 production by IL-2-deprived Th9 cells may require Rorγt. To initially test this possibility, we compared the transcriptomes of IL-2-deficient and sufficient Th9 cells against Rorγt-induced and repressed genes (sourced from (28)). Interestingly genes that were more highly expressed in IL-2-deficient compared to IL2-sufficient Th9 cells were highly enriched in Rorγt-induced but not repressed genes (Fig 5A). These data suggest a role for Rorγt in STAT3-independent IL-17 production.

To determine if Rorγt activity was required for STAT3-independent IL-17 production, WT and STAT3-deficient naïve CD4 T cells were isolated and cultured under Th9 and Th17

conditions as above. In some conditions, we also included the Rorγt-specific inhibitor Digoxin which interferes with Rorγt ligand binding and induction of transcription (35). While Th9 cells express high levels of Rorγt, Digoxin treatment did not significantly inhibit the production of IL-9 in these experiments or the expression of BATF or IRF4 in either Th9 or IL-2-deprived Th9 cell conditions (Fig 5B, C). Of note, addition of the vehicle control alone (DMSO) had an effect on the ability of STAT3-deficient IL-2-deprived Th9 cells to produce IL-17, likely due to the mild cytotoxic effects of DMSO on T cells (36). Nonetheless, between 8–16% of STAT3-deficient cells were capable of producing IL-17 in this setting (compare to ~0% in conventional STAT3-deficient Th17 conditions, see Fig 3A). Importantly, addition of Digoxin or TMP778 (another Rorγt inhibitor, (37)) to both WT and STAT3-deficient IL-2-deprived Th9 cultures virtually abolished IL-17 production in these settings (Fig 5B, C). As previously described (35, 37), neither Digoxin nor TMP778 altered Rorγt protein expression and we further demonstrate here that abrogation of Rorγt activity with digoxin or TMP778 did not alter the expression of the Th17-associated "accessory" factors BATF or IRF4 (Fig 5C). Together, these data indicate that Rorγt activity was required for STAT3-independent IL-17 production by Th cells and this was not associated with a loss of BATF or IRF4 expression.

BATF drives IL-17 production by IL-2-deprived Th9 cells

BATF is required for both Th9 and Th17 differentiation and also binds enhancer elements near the *Il9* locus (16, 17) and in the *Il17* promoter (28). Similar to Rorγt, BATF expression in IL-2-deprived Th9 cells, but not Th17 cells, was also maintained in the absence of STAT3 (see Fig 3C-E), suggesting that BATF may also play a role in STAT3-independent IL-17 production. We demonstrate here that BATF-deficient cells exhibit dramatic reductions of IL-9 and IL-17A production under both IL-2-deprived Th9- and Th17-skewing conditions (Fig 6A, B). Interestingly, while BATF was required for optimal IL-17F production in Th17 cells, it was not required in IL-2-deprived Th9 polarizing conditions (Fig 6B) suggesting that other factors, potentially Rorγt, can promote IL-17F production even in the absence of BATF. Indeed, Rorγt protein expression was similar in WT and BATF-deficient IL-2-deprived Th9 cells, whereas conventional Th17 cells required BATF for optimal Rorγt expression under IL-2 neutralizing and non-neutralizing conditions (Fig 6C, D). Together, these data suggest that Rorγt and BATF play non-redundant roles in STAT3-independent IL-17 production by Th cells.

Ectopic expression of Rorγt and BATF partially rescues IL-17 production in STAT3-deficient Th17 cells.

Our data above indicate that expression of Rorγt and BATF are STAT3-independent in IL-2-deprived Th9 cells and are required for IL-17 production in this setting. In contrast, Rorγt and BATF expression are dramatically reduced in STAT3-deficient Th17 culture conditions (see Fig 3). Based on these data we hypothesized that restoration of Rorγt and BATF expression could rescue IL-17 production in STAT3-deficient Th17 cells. To test this hypothesis, we transduced WT and STAT3-deficient Th cell cultured under conventional Th17 conditions with retroviruses expressing Rorγt-GFP and BATF-Thy1.1 (Supp Fig 3A) and examined IL-17 production after 4 days of culture. In WT cells, single transduction with Rorγt or BATF-expressing retroviruses enhanced IL-17 production, with ectopic

Rorγt expression having the dominant effect. Transduction with both factors resulted in an additive increase in IL-17A and F production (Supp Fig 3A-C). In STAT3-deficient cells, however, transduction with Rorγt or BATF alone had minimal effect on IL-17A or F production. On the other hand, STAT3-deficient cells with ectopic expression of both factors exhibited enhanced IL17A/F production to levels seen in control-transduced WT cells, but ~4–5-fold less than WT cells transduced with both Rorγt and BATF. This difference in IL-17 production was not due to differential expression of Rorγt and BATF in WT and STAT3-deficient cells after transduction, as Rorγt and BATF protein (as measured by the associated HA tag) levels were virtually identical (Supp Fig 3C). Taken together, these data indicate that at least part of STAT3's role in promoting the IL-17 production is through the induction of Rorγt and BATF.

Discussion

While STAT5-mediated gene transactivation clearly plays an important role in the differentiation of Th9 cells, its role as a transcriptional repressor in this lineage is also critical. As a transcriptional repressor, STAT5 has been previously shown to directly bind and limit the expression of the TFH-associated factor BCL6 that competes for STAT5 binding sites within the *Il9* promoter (12, 13). However, our recent work indicated that WT and BCL6-deficient Th9 cells exhibited very similar capacities to differentiate into IL-9 producing cells under optimal conditions (27), indicating that STAT5 may have other repressive activities outside of regulating BCL6 expression.

Here we demonstrate that STAT5 activity is also associated with the development of IL-9-producing Th cells in human allergic disease. These data are consistent with previous reports indicating that $CD4^+$ $CCR4^+$ cells, known IL-9 producers in human allergic airway disease, exhibit enhanced STAT5 phosphorylation in allergic vs non-allergic patients. Further, STAT5 activity in these cells correlated with increased chromatin accessibility at *IL9* promoter and enhancer regions (16). While a number of STAT5 target genes (i.e. *IL5, IL9, IL13, GATA3, IL1RL1*) were induced in human Th9 cells during an HDM-driven allergic response ((22), Fig 1, Supp Fig 1D-E), a STAT5-mediated suppressive signature was also prevalent in human *IL9*^{hi} cells as compared to *IL9*^{lo} "Th2" cells (Fig 1), suggesting that STAT5-mediated gene repression may also contribute to human Th9 cell development. Interestingly, *IL9*^{lo} cells that exhibited a lesser STAT5-repressed gene signature and an elevated Th17-like signature which suggests that STAT5 may regulate the balance of a Th9 vs Th17-like phenotype cells during human allergic disease.

In our studies, we observed increased IL-17 mRNA and protein expression by Th9 cells cultured in an IL-2-limiting environment (Fig 2). While a number of Th17-associated transcripts (i.e. *Rora*, *Rorc*, *Ccr6*) were increased in the absence of IL-2, these cells did not adopt a canonical Th17- or STAT3-associated transcriptional profile (Fig 2–3). Instead, these data suggest an alternative pathway of IL-17-producing cell differentiation. In support of this, IL-17-producing cells from IL-2-deprived Th9 conditions developed in the absence of the key Th17-associated factor STAT3 (Fig 3). Interestingly, innate-like T cells (i.e. γδ T cell receptor $(TCR)^+$, invariant natural killer T cells and innate-like αβ T cells) also exhibit a similar STAT3-independent IL-17 pathway (38–40). Whereas innate lymphoid cells (i.e.

non-TCR-expressing cells) maintain a requirement for STAT3 for the production of IL-17 (41, 42). As ILCs lack the capacity to receive signals through the TCR, this may suggest that TCR signaling compensates for lack of STAT3 in IL-17 production. Indeed, innate-like αβ T cells produced IL-17 only in response to TCR engagement in the presence of IL-1 (38). Likewise, STAT3-independent IL-17 production by IL-2-deprived Th9 cells in our studies was optimal in cell continuously cultured on anti-CD3-coated plates and was diminished in cells that were rested prior to restimulation (data not shown). Thus, TCR signaling is likely a critical factor that drives STAT3-independent production of IL-17 by all TCR^+ cells.

In Th17 cells, STAT3 signaling induces the expression of Rorγt and BATF, which in turn, are required for Th17 cell differentiation (28, 31). In our studies, Th9 cells also expressed surprisingly high levels of Rorγt and this expression was further enhanced upon IL-2 blockade (Figs 2 and 5). In contrast to canonical Th17 cells, Rorγt and BATF expression was STAT3-independent in IL-2-deprived Th9 cells and ablation of these factors dramatically reduced IL-17A production under IL-2-deprived Th9 polarizing conditions. These data suggest that while IL-17 production under these conditions is STAT3-independent, it maintains a requirement for Rorγt and BATF. Interestingly, STAT3-independent production of IL-17 by essentially all innate-like T cells is associated with or requires expression of Rorγt (38, 43). However, the role of BATF in IL-17 production by innate-like T cells is more controversial. BATF, and its transcriptional co-factor IRF4, are dispensable in for IL-17 production by certain subsets of $\gamma\delta TCR^+$ cells (43, 44). In contrast, BATF, but not IRF4, is required for development of IL-17-producing NKT cells (45, 46). Given that IL-17 is critical for the antimicrobial response at anatomical barrier sites (i.e. skin, intestines, eyes, lungs), the evolution of multiple IL-17-inducing pathways likely ensures protection of these susceptible organs.

The data above indicate that multiple subsets of innate-like T cells can produce IL-17 in the absence of STAT3, and our data demonstrate that adaptive Th cells have similarly retained this capacity. Despite this, STAT3-independent production of IL-17 by Th cells is not commonly observed in vivo (31). One potential explanation for this may be the multiple roles of STAT3 in promoting Th cell survival/proliferation in vivo. STAT3 has been long known for its ability to promote Th cell survival by directly binding and inducing anti-apoptotic and proliferative factors (i.e. *Bcl2*, *Ier3*, *Fos*, *Fosl2*) (33). Further, recent data in Th17 cells demonstrates that STAT3 suppresses the anti-proliferative functions of STAT1 and maintains mitochondrial membrane potential in established Th17 cells (30). STAT1 hyperactivation in cells with a STAT3 loss of function mutation also leads to induction of PD-L1 which inhibits IL-17 production in vivo (47) and may also influence Th cell survival. We demonstrated here that while STAT3 was not required for the initial differentiation of IL-17-producing cells in IL-2-deprived Th9 cultures, it was required for their prolonged survival after multiple rounds of differentiation (Supp Fig 2). These data may explain why STAT3-independent IL-17-producing Th cells are not observed in the steady state intestines, mouse models of disease or in patients with STAT3 loss of function mutations (34, 48).

Work by our group and others have noted the relative instability of Th9 cells in culture (32), after adoptive cell therapy (3, 49) and in murine disease models (50). In culture, we demonstrated that Th9 cells rapidly lose their capacity to produce IL-9 upon secondary

culture and gain the capacity to produce IL-10 and IL-17 (32). While the loss of IL-9 was associated with increased STAT3 activation, blockade of IL-10-induced STAT3 in these cultures only moderately rescued IL-9 production and did not impair the transition to an IL-17-producing phenotype (32). Of note, this loss of IL-9 and gain in IL-17 production correlated with loss of responsiveness to IL-2 (unpublished data). We extended these findings here to show that STAT3 was also not required for the generation of IL-17-producing cells after long-term culture of Th9 cells (Fig 4). These data indicate that when IL-2 is limiting, through either IL-2 blockade or natural decay of bioavailable IL-2, this favors the differentiation of STAT3-independent IL-17-producing Th cells. As a whole, these data indicate a role for the transcriptional inhibition by IL-2/STAT5 for maintenance the Th9 phenotype and that this occurs at least partially through limiting the emergence of a Th17-like differentiation program. Further, our data suggest that STAT5 transcriptional inhibition is a key player in Th9-driven immunopathology in human allergic disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors would like to thank Mr. Sungtae Park and Ms. Nicole Anderson for their helpful comments on this manuscript and Ms. Tripti Bera for her technical assistance that made this work possible.

This work was supported by a Purdue University Ross-Lynn Graduate Student Fellowship to D.A.C. A NIDDK R01DK120320 to D.L.B. and an NIGMS R35GM138283 to M.K. M.R.O was supported by Purdue University startup funds (Purdue University) and a Showalter Trust Award (41000747).

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Key Points:

1. Human Th9 cells exhibit a STAT5-mediated gene suppression signature.
2. IL-2/STAT5 suppresses a STAT3-independent Th17-like phenotype in Th9 cells.
3. IL-17 production by IL-2-deprived Th9 cells requires Rorγt and BATF.

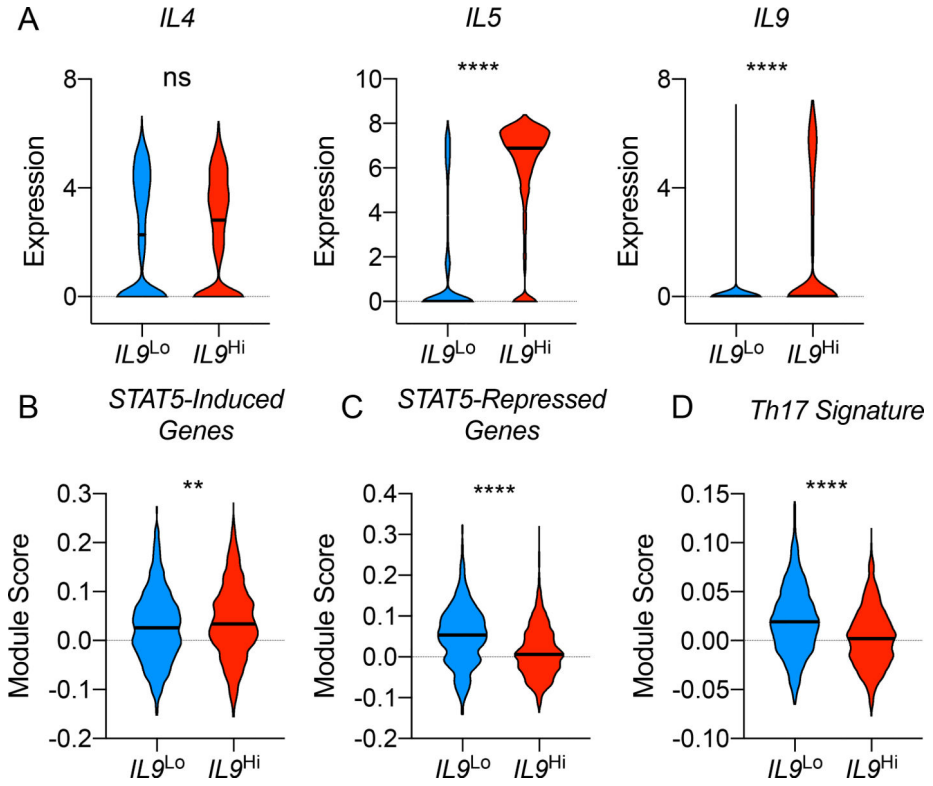

Figure 1. STAT5 signatures in *IL9*-expressing Th cells during the human allergic response.

(A) Expression of *IL4*, *IL5*, *IL9* genes in human pulmonary *IL9*^{hi} and *IL9*^{lo} cells in house dust mite allergic patients (22). (B-C) Module scores for Th cell-associated STAT5-induced (B) and repressed (C) genes in *IL9*^{hi} and *IL9*^{lo} cells. (D) Module scores for Th17-associated genes in *IL9*^{hi} and *IL9*^{lo} cells.

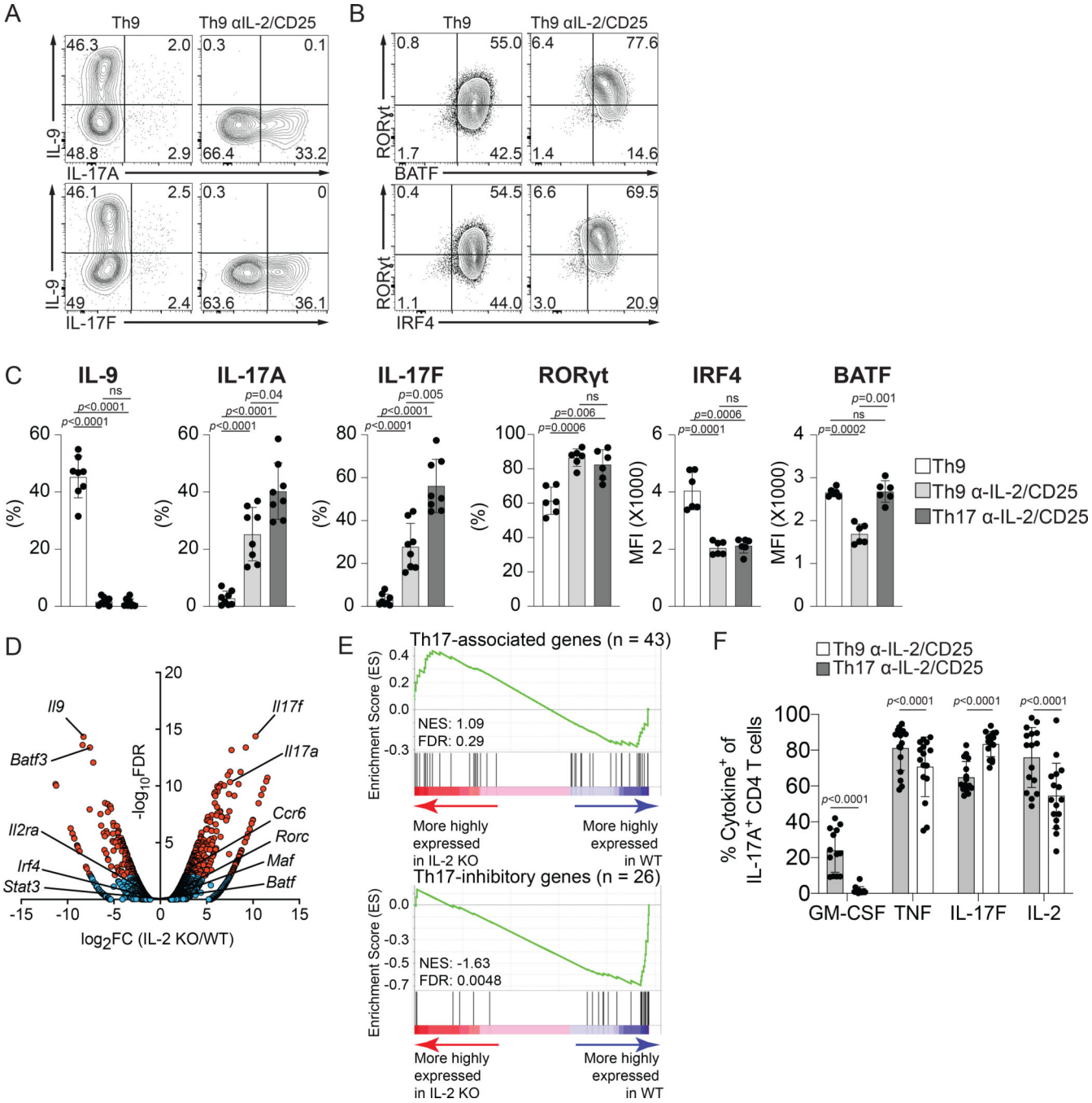

Figure 2. IL-2 suppresses a Th17-like phenotype in differentiating Th9 cells.

(A) Intracellular IL-9, IL-17A and IL-17F protein expression of naïve CD4 T cells cultured under Th9 conditions with and without IL-2 neutralization. (B) Representative flow cytometry contour plots for RORγt, BATF and IRF4 transcription factor expression of Th cells cultured as per above. (C) Compiled cytokine and transcription factor data from in vitro polarized CD4 T cells, each data point represents one mouse. These data represent cells from at least n=6 mice. (D) Volcano plot showing mean gene expression values (n=3) in IL-2-sufficient (WT) and -deficient (KO) Th9 cells reanalyzed from previously

published RNA-seq data (13). Select genes associated with Th9 or Th17 differentiation are highlighted. (E) Gene Set Enrichment Analysis (GSEA) comparing transcriptomes of IL-2-deficient (KO) and sufficient (WT) Th9 cells against indicated gene set on top. NES: normalized enrichment score. FDR: false discovery rate. Indicated gene sets were sourced from (51) and are included in Supp Table 1. (F) Intracellular flow cytometry analysis of cytokine co-expression by IL-17A-producing cells cultured under IL-2-deprived Th9 or Th17 conditions. These data represent at least n=14 mice. Paired *t*-test p-value <0.05 was considered significant.

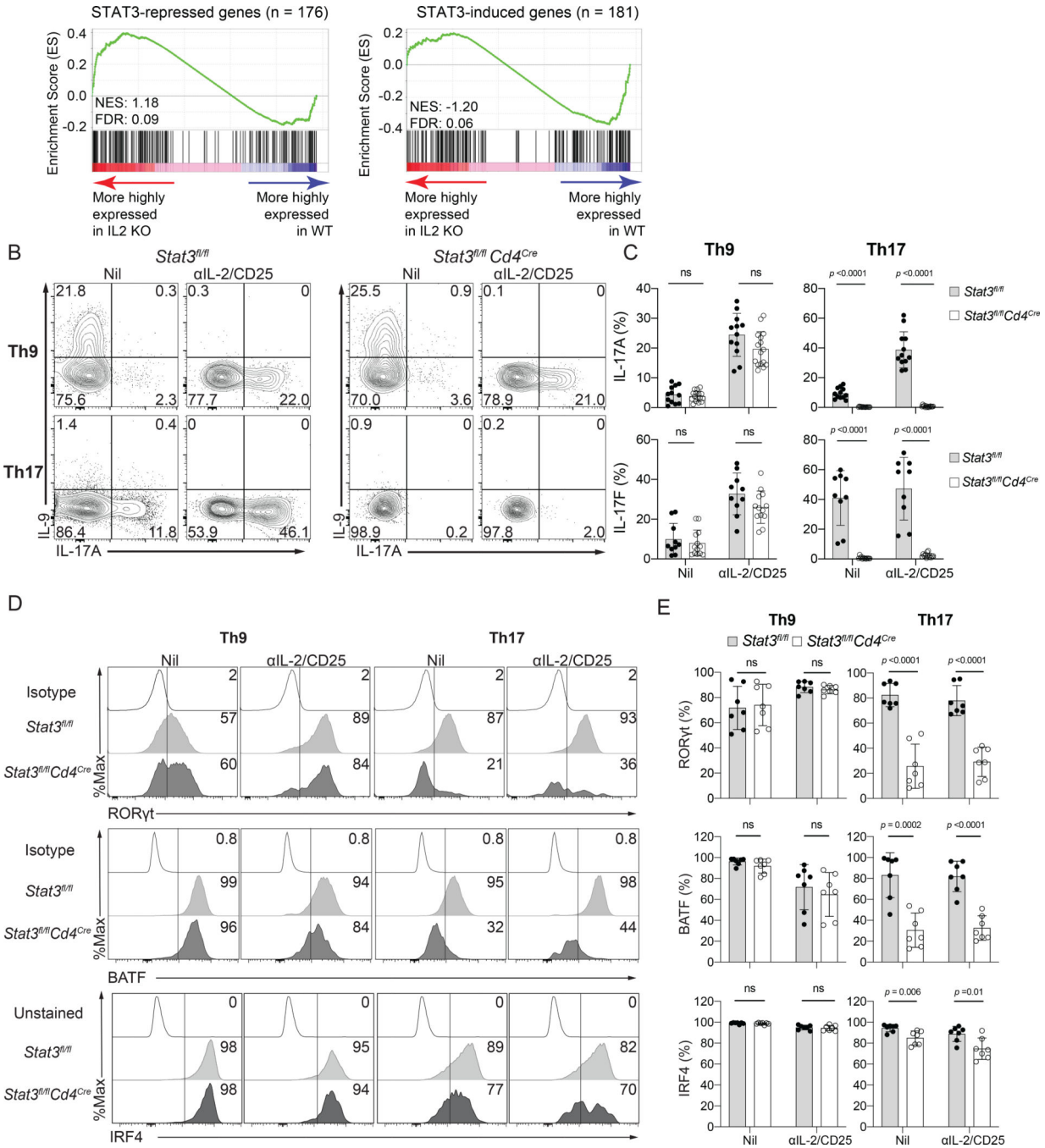

Figure 3. STAT3-independent IL-17 production by IL-2-deprived Th9 cells.

(A) GSEA comparing transcriptomes of IL-2-deficient (KO) and -sufficient (WT) Th9 cells against indicated STAT3- repressed and induced gene sets NES: normalized enrichment score. Indicated gene sets were sourced from (28) and are included in Supp Table 1. WT and STAT3-deficient naïve CD4 T cells cultured under Th9 and Th17 conditions with and without IL-2/CD25 blockade. (B) Representative data of IL-17 production in the conditions mentioned. (C) Compiled data of multiple experiments, each data point represents one mouse. In this panel, we represent at least n=9 individual mice per group. (D) Representative

histograms of the transcription factors expression pattern in the T cells cultured under the conditions previously mentioned. (E) Quantified transcription factor staining data. Nil = no antibody treatment. Each data point represents one mouse. n=7 per group. Unpaired t-test p-value <0.05 was considered significant.

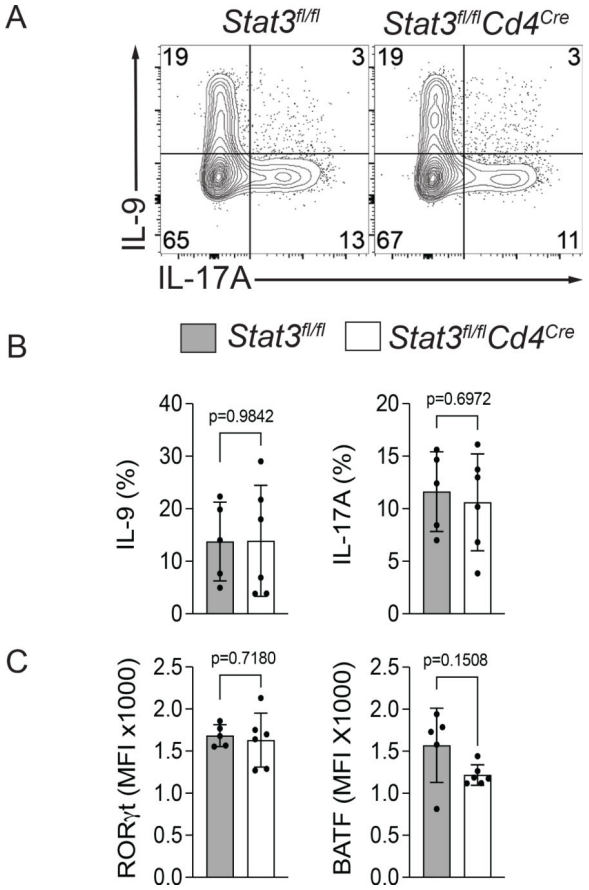

Figure 4. Prolonged culture of Th9 cells results in STAT3-independent IL-17 production.

Naïve CD4 T cells were isolated from the spleens of *Stat3*$^{fl//fl}$ and *Stat3*$^{fl//fl}$ *Cd4*-Cre mice and were cultured under standard Th9 polarizing conditions for 4 days. On day 4, cells were collected and transferred to fresh non-anti-CD3-coated plates with ½ concentration of original cytokines. After this "rest" period, cells were recultured at 10^6 cells/ml and in these same conditions with plate-bound anti-CD3 for an additional 4 days prior to harvest. A) Representative contour plots of IL-9 and IL-17 production by Th cells under each condition. B) Cumulative frequencies of IL-9 and IL-17 positive Th cells. C) Rorγt and BATF expression (MFI, mean fluorescence intensity) in cultured cells. n=5 for each analysis and p values are derived from unpaired t-tests.

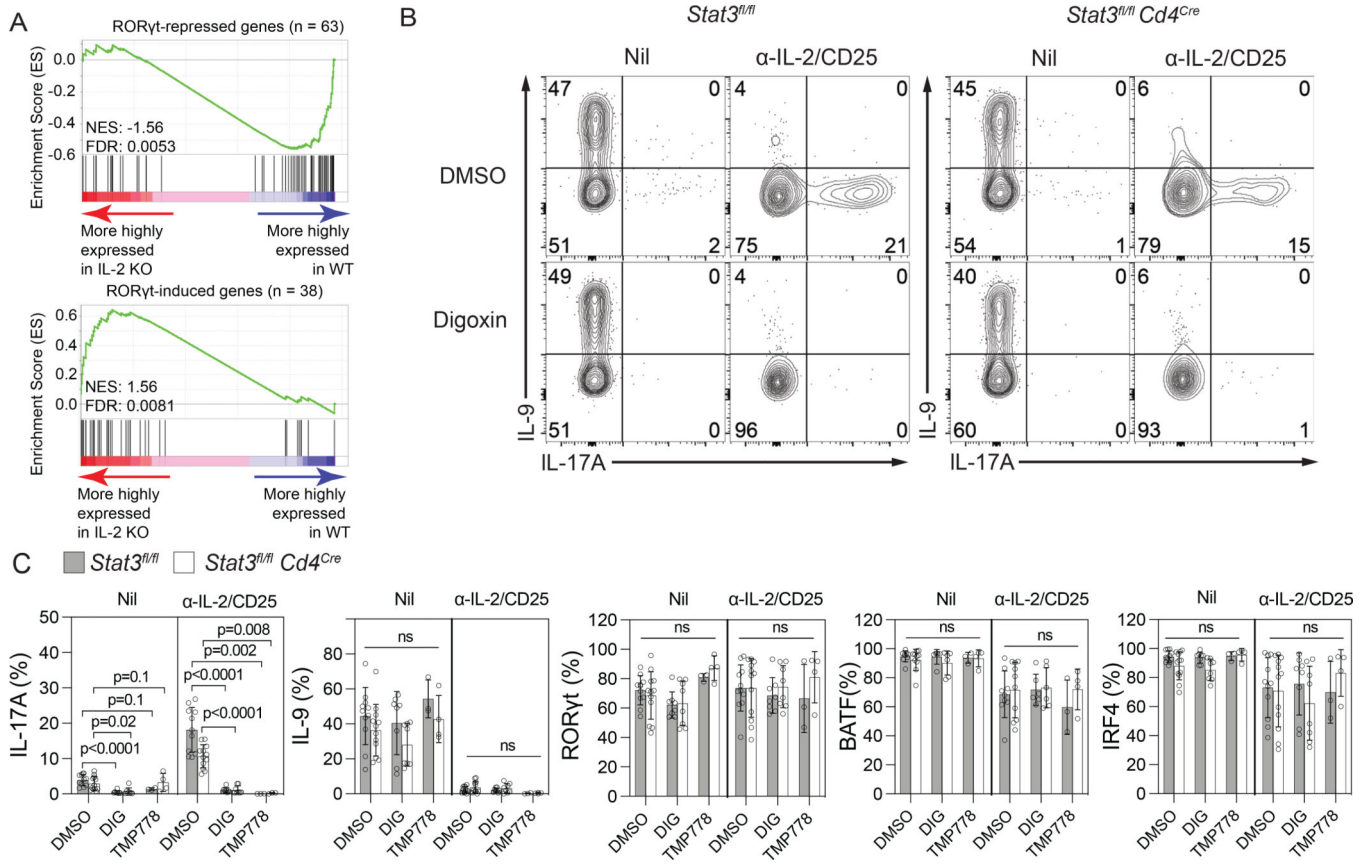

Figure 5. Rorγt activity is essential for STAT3-independent IL-17 production by IL-2-deprived Th9 cells.

(A) GSEA plots comparing transcriptomes of IL-2-deficient (KO) and sufficient (WT) Th9 cells against Rorγt-repressed (top panel) and -induced (bottom panel) genes. NES: normalized enrichment score. Indicated gene sets were sourced from (28) and are included in Supp Table 1. WT or STAT3 deficient naïve CD4 T cells were cultured in vitro under Th9 conditions (w or w/o anti-IL-2) and treated with either 10μM Digoxin or DMSO. Intracellular cytokine production and TF expression were assessed using flow cytometry. (B) Representative data of IL-17 production and RORγt expression of WT or STAT3-deficient T cells. (C) Quantified data of cytokine production and TF expression by Th cells cultured as above with Digoxin or TMP778. Nil = no antibody treatment. Each data point represents one mouse. n=8 per group. Paired t-test p-value <0.05 was considered significant

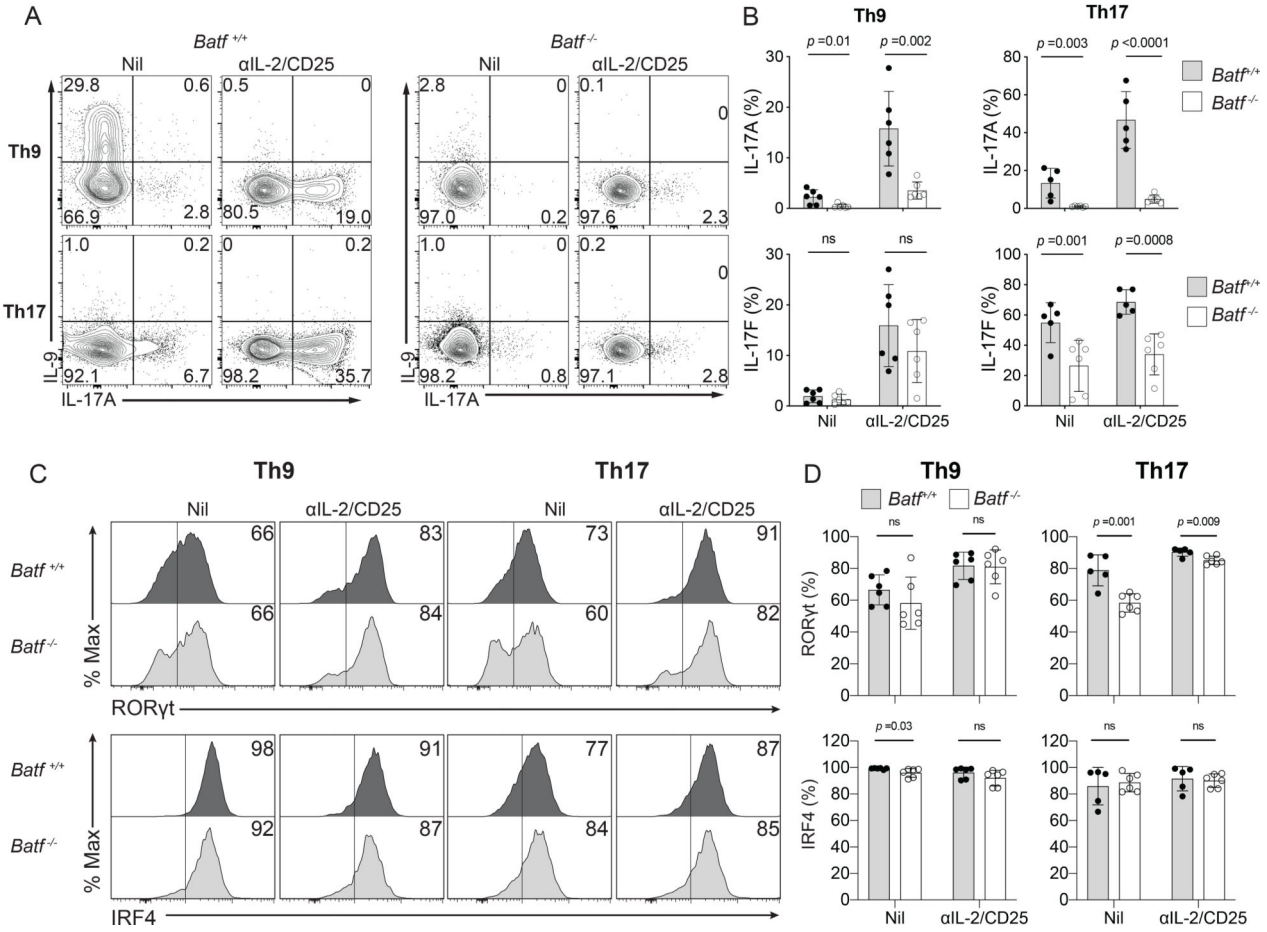

Figure 6. BATF is required for IL-17 production in IL-2-deprived Th9 cells.

WT and BATF-deficient naïve CD4 T cells cultured under Th9 and Th17 conditions with and without IL-2/CD25 blockade. (A) Representative data of IL-17 production in the conditions mentioned. (B) Compiled data of multiple experiments, each data point represents one mouse n=6 mice per group. (C) Representative data of the transcription factors expression pattern of T cells cultured as per above. (D) quantified transcription factor staining data. Nil = no antibody treatment. Each data point represents one mouse n=6 mice per group. Unpaired t-test p-value <0.05 was considered significant.