

Accepted Manuscript

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PII: S0091-6749(18)31001-7

DOI: [10.1016/j.jaci.2018.06.036](https://doi.org/10.1016/j.jaci.2018.06.036)

Reference: YMAI 13519

To appear in: *Journal of Allergy and Clinical Immunology*

Received Date: 19 December 2017

Revised Date: 20 June 2018

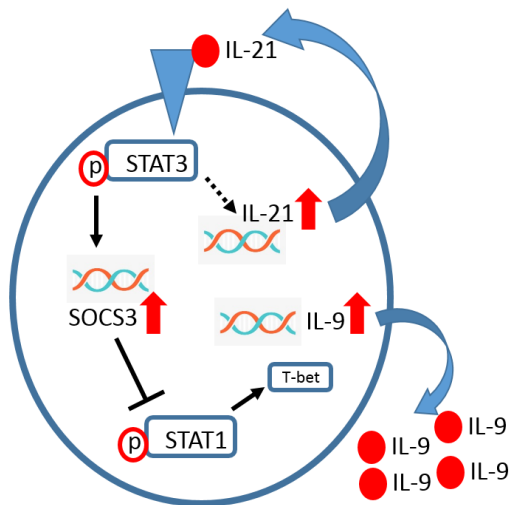
Accepted Date: 28 June 2018

Please cite this article as: Zhang Y, Siegel AM, Sun G, Dimaggio T, Freeman AF, Milner JD, Human Th9 differentiation is dependent upon STAT3 to restrain STAT1-mediated inhibition, *Journal of Allergy and Clinical Immunology* (2018), doi: 10.1016/j.jaci.2018.06.036.

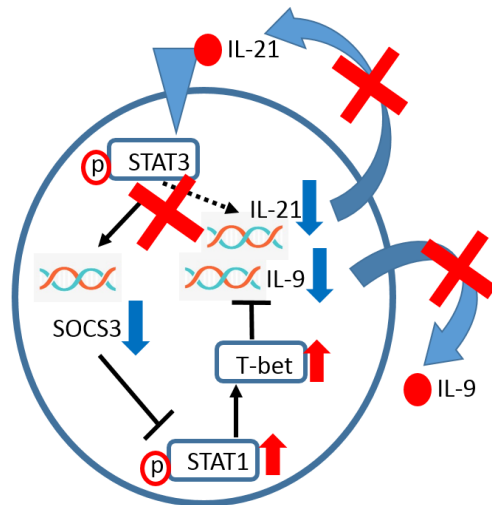
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STAT3 in Human Th9 Differentiation



Healthy
Th9



STAT3LOF
Th9

LOF: loss of function
STAT: Signal transducer and activator of transcription
SOCS: Suppressors of cytokine signaling
IL: Interleukin
Th: T helper

Human Th9 differentiation is dependent upon STAT3 to restrain STAT1-mediated inhibition

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This work was supported by the intramural research program of the National Institutes of Health 1ZIA AI001098-02.

The authors declare no additional competing financial interests.

21

22 **Abstract:**

23 **Background:** Patients with loss of function (LOF) signal transducer and activator of
24 transcription 3 (STAT3) mutations have dermatitis, enhanced IgE production despite a relative
25 lack of immediate hypersensitivity, recurrent infection, and an increased rate of lymphoma, in
26 addition to a number of skeletal and connective tissue abnormalities. Gain of function (GOF)
27 STAT1 mutant patients also have susceptibility to candidiasis and sinopulmonary infection, as
28 well as autoimmunity and squamous cell carcinoma, in addition to even more broad phenotypes.

29 **Objective:** Because of the link between Th9 cells and allergic inflammation, autoimmunity and
30 anti-tumor surveillance, and because evidence shows either the role for STAT3 or STAT1 in Th9
31 differentiation conflicts, we wished to determine the status on this lineage of STAT1GOF and
32 STAT3LOF in humans.

33 **Methods:** We detected IL-9 levels and Th9 differentiation of STAT3LOF and STAT1GOF
34 patients, together with Th9 transcript factors, and partially rescued their deficiency in vitro by
35 adding cytokines they lacked or transfecting key molecules.

36 **Results:** We found that PBMCs or sorted naïve CD4⁺ T cells from STAT3LOF and STAT1GOF
37 patients had impaired Th9 generation/differentiation. STAT3 inhibition in normal Th9 cultures
38 diminished early IL-21 induction and late IL-9 production, while exogenous IL-21 enhanced Th9
39 differentiation even with STAT3 inhibition, by restoring SOCS3 expression and thus inhibiting
40 excessive p-STAT1 activation. Furthermore, exogenous expression of SOCS3 or either T-bet or
41 STAT1 RNAi in STAT3 LOF cells partially rescued IL-9 differentiation.

Conclusion: Collectively, these results suggest that human Th9 differentiation depends on normal p-STAT3 and IL-21 production to suppress p-STAT1 activation and T-bet transcription.

Key message:

- STAT3 LOF and STAT1 GOF patients have impaired Th9 production.
- IL-21 could partially restore the Th9 defect in vitro by activating SOCS3 and inhibiting the excessive p-STAT1 and downstream T-bet which were preventing Th9 differentiation in these patients.

Capsule Summary: STAT3 LOF or STAT1 GOF mutation patients have impaired Th9 differentiation, at least in part due to excessive STAT1-mediated T-bet induction. IL-21 can partially rescue patients' IL-9 secretion, providing a novel mechanism for its anticancer effects.

Key words: *human Th9; STAT1; STAT3; IL-21; anti-cancer; Allergy*

Abbreviations used:

LOF: loss of function

STAT: Signal transducer and activator of transcription

GOF: Gain of function

SOCS3: Suppressors of cytokine signaling

TGF- β : Transforming growth factor beta

IRF4: Interferon regulatory factor 4

TFH: T follicular helper

HC: Healthy control

TF: transcription factor

HIES: Hyper IgE Syndrome

ERBIN: ERBB2-interacting protein

Introduction

CD4⁺ helper T cells orchestrate adaptive immune responses. Naïve CD4⁺ T cells can differentiate into distinct effector subsets upon encountering varied cytokine milieus, together with TCR stimulation. One of these CD4⁺ T helper subsets, Th9, produce IL-9 (1), and T-cell derived IL-9 appears to be a critical contributor to the pathogenesis of allergic disease (2) autoimmunity (3), and even anti-tumor activity (4, 5).

Th9 cells can be generated in vitro by supplementing typical Th2 differentiation conditions—which include IL-4-- with TGF- β 1, which, via STAT6 and Smad signaling, promote the expression of Th9-regulating transcription factors IRF4 and PU.1 respectively(2, 6-9). We have previously shown that TGF- β signaling is enhanced in STAT3LOF T-cells, therefore raising the question as to whether this might impact Th9 differentiation in these patients (10). Furthermore IL-21, a Th17 and TFH-associated cytokine which can signal via STAT3, is also produced under Th9 polarization (3, 11), and yet the role of IL-21 and STAT3 in Th9 differentiation and function is still unclear. Some mouse models have shown that IL-21 can inhibit Th9 differentiation (12), and that STAT3 deficiency in mice can increase Th9 differentiation(13), while others have either shown no effect (7), or actual enhancement of Th9-mediated anticancer effects by IL-21 (11). Other STAT3 activating cytokines, such as IL-23, have been reported to inhibit Th9 (3, 5), and adding IL-6 in Th9 culture also inhibits IL-9 production by impairing p-STAT5 (14). Human Th9 induction can be augmented by adding IL-21, as well as IL-6 (15), and ex vivo candida specific IL-9 production is reduced in patients with STAT3 loss of function mutations (16).

Counterregulation of STAT functions by different STAT proteins ensures specificity of cellular lineages and fates, and understanding of this counterregulation could help shed light on Th9 differentiation, as well as related patients' pathogenesis and treatment. Suppressors of cytokine signaling (SOCS) proteins are induced by particular cytokines and regulate the p-STATs and other signals via a negative feedback loop (17). We and others have shown naïve T cells from patients with STAT3 LOF have reduced *SOCS3* transcripts (18, 19), and such deficiency causes excessive p-STAT1 levels both under cytokines stimulation and on terminal differentiated Th subsets (20). IL-27 can inhibit Th9 differentiation partially through a STAT1 dependent, not STAT3 dependent pathway (21), by enhancing T-bet expression. T-bet is also reported to inhibit IL-9 production in innate lymphoid cells (ILCs) in lung (22). On the other hand, STAT1 $-/-$ CD4 $^{+}$ T cells show impaired IL-9 and IL-21 production under Th9 condition with IL-1 β , as STAT1 is reported to increase IL-9 production through binding to *Irf1* promoter and promoting *Irf1* expression (11). In humans, both IFN γ and IL-27 significantly inhibit IL-9 production(15), and patients with STAT1 gain-of function mutations have fewer ex vivo candida specific Th9 cells, all suggesting STAT1 suppresses human Th9 differentiation (16).

Patients with mutations leading to STAT1GOF and STAT3LOF provide an opportunity to better dissect the consequences of disruptions to the STAT pathways in Th9 differentiation. Further, the enrichment of neoplasms in both diseases (23), coupled with autoimmunity in STAT1GOF patients (23-26) and the relative protection from atopic disease in STAT3LOF despite high IgE (19, 27), raises the question as to how Th9 cells might contribute to these disorders.

Here we found that both STAT3LOF and STAT1GOF patient cells have impaired Th9 differentiation and ex vivo IL-9 production. This appears to be due at least in part to excessive

STAT1 activity leading to T-bet mediated IL-9 inhibition. IL-21, by enhancing SOCS3 production and inhibiting STAT1 activity during differentiation, can partially rescue Th9 differentiation in vitro.

Methods

Human subjects and clinical studies

All patients or their guardians, along with healthy family members who did not have causal mutations provided informed consent under approved protocols of the National Institute of Allergy and Infectious Diseases Institutional Review Board 10-I-0148 and 00-I-0159. Patients with STAT3LOF or STAT1GOF all had validated known and/or published causal mutations and blood was drawn during visits for routine care. Additional genotype and phenotype information is included in Table S1. Blood from healthy controls not obtained via 10-I-0148 was obtained from the NIH Blood Bank.

Th9 differentiation and cytokine analyses

PBMC were isolated from whole blood by Ficoll-Hypaque gradient centrifugation. Naïve CD4⁺ T cells were isolated from PBMC using negative selection magnetic activated cell sorting microbeads (Miltenyi Biotec) as per manufacturer's protocol. Purity was >95% in all samples run. PBMC or naïve cells were stimulated at 500,000 cells/well in 48-well plates that had been coated with anti-CD3 (1 µg/mL, OKT3 eBioscience) antibodies. The cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (GIBCO) supplemented with 10% heat-inactivated FCS (Gemini Bio-Products), 100 U/mL penicillin G, 100 µg/mL streptomycin, and 29.2 µg/mL L-glutamine (all GIBCO), at 37° C/5% CO₂ for 5 days in the presence of soluble anti-CD28 (0.5 µg/mL, BD Pharmingen). For Th9, anti-IFNγ (10 µg/mL, BD Pharmingen), IL-4 (30 ng/mL, R&D), IL-1β (10 ng/mL, Peprotech), and TGF-β (5 ng/mL, Peprotech) were added

in the culture. For Th0, 100 U/ml IL-2 (R&D) was added. For Th2, anti-IFN γ (10 μ g/mL, BD Pharmingen), anti-IL10 (10 μ g/mL, BD Pharmingen), IL-4 (30 ng/mL, R&D), IL-2 (100 U/mL, R&D) were added. In certain experiments, cells were cultured with IFN γ , IL-6, IL-10, IL-12, IL-21, IL-23, IL-27 (all 10ng/ml, R&D), or pre-treated with 10 μ g/ml of anti-human PD-L1 monoclonal antibody (AMP-714; kindly supplied by Amplimmune, Inc.), UltraLEAF purified human isotype control IgG4 antibody (BioLegend), with Th9 polarization. STAT3 antagonist C188-9 was used in some experiments and been described (28).

Intracellular cytokine staining and other flow cytometry

On day 5, cells were stimulated with 20 ng/mL PMA and 1 μ M ionomycin for 5 hours. 10 μ g/mL brefeldin A was added after 2.5 hours. Cells were then fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences) as per manufacturer's protocol, and stained with Live/Dead Fixable Aqua dye, anti-human CD3, CD4, IL-9, IL-4, p-STAT1, p-STAT5, GATA3, and anti-mouse CD3 and IL-9 (BD Biosciences), anti-human CD45RO ECD (Beckman Coulter) and anti-human IL-21, T-bet, and anti-mouse CD4 CD44 IL-4 (eBiosciences) antibodies. For SOCS3 staining, DyLight 488 Anti-Mouse IgG (Abcam) was used following anti-SOCS3 (abcam). PU.1 (BioLegend) and GATA3 (BD Biosciences) antibodies were used for TF staining, with Foxp3 / Transcription Factor Staining kit (eBioscience), follow manufacturer's protocol. Events were collected on an LSRFortessa with Diva 6.1.3 software (BD Biosciences) and analyzed with FlowJo 10 software (Treestar, Ashland, OR). All plots are gated on live singlet CD3⁺ CD8⁻ CD45RO⁺. All values used for analyzing proportionate responses are background-subtracted.

Real-time PCR

Cells were then washed and pelleted. Total RNA was extracted from PBMC with the RNeasy Mini Kit (Qiagen). For real-time PCR, 1 µg of total RNA was reverse transcribed (Invitrogen) and the resulting cDNA was amplified by means of PCR with the ABI 7500 Sequencer and TaqMan expression assays (Applied Biosystems). 18S was used as a normalization control. The data were analyzed with the $2^{-\Delta\Delta CT}$ method, and results are expressed as mean fold induction.

SOCS3 transfection

Naïve cells after 48h of anti-CD3/CD28 stimulation were transfected with either GFP control (Lonza) or 5µg SOCS3-GFP construct (OriGene) using Amaxa Human T-cell Nucleofection Kit (Lonza) following manufacturers protocol, using Amaxa nucleofection program U-014. Cells were cultured under Th0 or Th9 condition for 5 days after transfection. For p-STAT5 analyzing, 24h after transfection, cells were harvested and stimulated with 80ng/ml IL-2 for 30m, and p-STAT5 levels on CD4⁺ cells were detected.

siRNA transfection

PBMC or Naïve cells after 48h of anti-CD3/CD28 were transfected with 30pM *STAT1* or *TBX21* siRNA (ThermoFisher Scientific) using Amaxa Human T-cell Nucleofection Kit (Lonza) per manufacturer's protocol. Control cells were transfected with 30pM nonspecific, scrambled siRNA. Cells were cultured under Th0 or Th9 condition for 5 days after transfection.

Stat3-mut HIES mouse and mouse Th9 differentiation

All animal work was performed under an Animal Care and Use Committee–approved protocol. All mice were derived from a C57BL/6 background. Littermates are obtained by breeding from the original sources, and no outside WT mice were used as controls or for breeding. The Stat3-mut mouse was a gift from John O’Shea (NIA MS, NIH, Bethesda, MD). The mouse has a single aa deletion ($\Delta V463$) in the STAT3 DNA-binding domain to mimic the heterozygous mutation seen in patients (29). Naïve cells were isolated from splenocytes and were cultured in RPMI 1640 with 10% FBS, 2-ME, and were activated with plate-bound anti-CD3 (1 ug/ml, 2C11, Biolegend) and soluble anti-CD28 (1 ug/ml, 37.51, BD Biosciences) and cultured under Th9 (IL-4 20 ng/ml, PeproTech; TGF- β 5 ng/ml, PeproTech), or Th0 conditions (IL-2 1ng/ml, PeproTech) in a 48-well plate.

Results

Decreased Th9 levels and Th9 transcript factors expression in STAT3LOF and STAT1GOF patient cells.

To assess the frequencies of short term induced Th9 cells, PBMC from healthy controls (HC), and subjects with either STAT1GOF or STAT3GOF were stimulated with anti-CD3/CD28 for 3 days in the presence of Th9 inducing/expanding cytokines. STAT1GOF and STAT3LOF IL-9 production was substantially lower than the healthy controls. (Fig. 1A) IL-21 production was also impaired in STAT3LOF and, to a lesser extent, STAT1GOF (Fig. 1A B), while both STAT3LOF and STAT1GOF PBMC had increased IL-4 production under these conditions (Fig. 1B). To determine whether the reduced frequency of Th9 cells resulted from poor differentiation from naïve precursors, we isolated naïve CD4 T-cells from subjects and controls and placed them under Th9 polarizing conditions for 5 days. IL-9 production was indeed lower in STAT3LOF and STAT1GOF samples compared to HC (Fig 1C D E). Similarly, IL-21 induction was lower in STAT3LOF cultures, compared to STAT1GOF and HC, while IL-4 induction was increased compared to control in STAT3LOF under Th2, but not Th9 condition (Fig 1C D). Expression of IRF4, and GATA3, both Th9 associated TF downstream of IL-4/p-STAT6, were normal, consistent with the normal p-STAT6 activation observed under Th9 conditions (Fig. 1 E, Fig. S1A, Fig. S1B). PU.1, another Th9 related TF downstream of TGF- β , was highly expressed in PBMC from STAT3LOF patients (Fig. 1 F), consistent with our previous data showing patients with STAT3LOF have enhanced TGF- β responses (10).

Taken together, we found CD4 T-cells from patients with STAT1GOF and-- in contrast to mouse models-- STAT3LOF, have a significant IL-9 production deficiency both in PBMC

(reflecting, at least in part, a lack of existing Th9 cells) and after naïve T-cell differentiation conditions, while a focal diminution of IL-21 expression was observed in STAT3LOF. This defect occurred despite the normal PU.1 expression in STAT3LOF PBMC., implying that other factors prevent normal IL-9 production in STAT3LOF and STAT1GOF mutant patients.

P-STAT3 signal blocking cause IL-21 disappearance and IL-21 can restore abnormal IL-9 levels in STAT3LOF and STAT1GOF patient cells in vitro.

To establish whether acute STAT3 inhibition-- vs. congenital loss of function in STAT3-- could recapitulate our results, we used a small molecule inhibitor of STAT3, C188-9 in naïve Th9 differentiation cultures of HC over 8 days. The IL-9 defects were similar to those using primary patient cells (Fig. 2A-C), while IL-4 was highly increased under low concentration of inhibitor (Fig. 2D). We noted that over the time course of differentiation, the difference in IL-21 production was notable prior to that of IL-9, raising the possibility that IL-21 might actually have a role in IL-9 regulation during differentiation when STAT3 signal is impaired, similar to that reported in healthy controls (15). We therefore added exogenous IL-21 into Th9 naïve differentiation culture conditions, and noted that STAT3LOF and STAT1GOF IL-9 expression could be partially rescued (Fig 2E-F). Interestingly, we noted that most HC with normal IL-9 production had no response to IL-21, however several, whose Th9 induction was on the lower end, responded to IL-21 with increases in IL-9 production. We also tested a series of other cytokines, and found that other STAT3 agonists-- IL-6, IL-23 could increase IL-9 levels in some

patients as well (Fig. S2 A B). IL-27 and IFN γ , which are strong activators of STAT1 as well, were inhibitory (Fig. S2 A B). We also found PU.1 levels were unaffected by IL-21 (Fig. S2 C), while IL-4 levels were decreased by adding IL-21, suggesting IL-21 also affects Th2 cytokine production (Fig. S2 D).

IL-21 restores IL-9 levels by inhibiting p-STAT1.

Because STAT1 agonist cytokines inhibited Th9 differentiation and both STAT3LOF and STAT1GOF T-cells exhibit enhanced pSTAT1 activation (20), we sought to measure pSTAT1 under Th9 conditions. We found that pSTAT1 was increased in STAT3LOF, in addition to the expected increase of pSTAT1 in STAT1GOF, when compared to HC on day 5 of culture. (Fig. 3A-B). Addition of IL-21 brought the pSTAT1 levels down, in bimodal fashion, in the STAT3LOF cultures (Fig. 3A-B). Of note, in the STAT3LOF patients, most IL-9⁺ and IL-21⁺ cells could be found in the p-STAT1^{low} population (Fig. 3C), strongly suggesting that pSTAT1 directly suppresses Th9 differentiation. (Fig. 3 B D). For STAT1GOF patients, pSTAT1 levels were also downregulated by adding IL-21, accompanied by partial IL-9 rescue, though not dependent on pSTAT1 staining intensity and no bimodal pSTAT1 activation was observed, likely due to the underlying genetic predisposition for enhanced pSTAT1. Similarly, because there were no substantial elevations in pSTAT1 in healthy control cultures, no bimodal peak was observed. Unlike samples from STAT3LOF patients, STAT1GOF Th9 cultures lead to relatively normal IL-21 expression (Fig. 1C D), however not enough to rescue Th9 differentiation.

IL-21 increases SOCS3 expression through p-STAT1 and T-bet

Poor SOCS3 upregulation in STAT3LOF can lead to a failure to suppress pSTAT1 (20), and indeed SOCS3 message and protein levels were low in STAT3LOF Th9 cultures (Fig. 4A). Addition of IL-21 enhanced SOCS3 expression in STAT3LOF cultures, potentially by acting via the residual STAT3 function in these patients' cells (30). SOCS3 levels were unaffected in STAT1GOF. STAT1 transcriptional target, T-bet, which antagonizes Th9 differentiation (21) was elevated in STAT3LOF and STAT1GOF Th9 cultures, and IL-21 partially reduced its expression. (Fig. 4B). We found IL-21 can also increase IRF4 levels in STAT3LOF (Fig. 4B). IRF4 is thought to be regulated by pSTAT6 however there was no difference in short term IL-4 induced pSTAT6 comparing patients and control cells (Fig. S1). The elevated PU.1 levels seen in STAT3LOF under Th9 differentiation conditions were not affected by IL-21 (Fig. 4B).

To recapitulate the mechanism of IL-21 rescue, we found that overexpression of SOCS3 in STAT3LOF and STAT1GOF suppressed T-bet levels (Fig. 4C) and partially rescued IL-9 mRNA levels (Fig. 4D), and partially restored IL-9 expression measured by intracellular staining (Fig. S3 A B)—albeit to a limited extent due to the profound effect SOCS3 overexpression has on cell function. Meanwhile, SOCS3 overexpression resulted in significantly lower IL-2–induced p-STAT5 in cells from HC and patients (Fig. S3 C). As p-STAT5 is actually a positive driver for IL-9 induction(31), it is unlikely that the effects of SOCS3 on IL-9 production in STAT3 LOF and STAT1 GOF patients' cells are via the STAT5 pathway. IRF4 mRNA was also increased by overexpression of SOCS3 (Fig. 4D). Furthermore, knockdown of STAT1 or T-bet in both STAT3LOF and STAT1GOF partially rescued Th9 levels (Fig. 4E F), and excessive T-bet levels in patients were effectively reduced by STAT1 and T-bet siRNA transfection, while IRF4 and

PU.1 remained unchanged (Fig. 4F). As anticipated, overexpression of T-bet in HC Th9 cultures suppressed Th9 expression (Fig. 4G). IL-21 therefore restores IL-9 expression when STAT1 activation is relatively high and STAT3 activation is relatively low through increasing SOCS3, inhibiting excessive p-STAT1 and T-bet.

In contrast to humans, STAT3mut mice cells have increased Th9 production.

In vitro cultures have shown that conditional deletion of Stat3 in CD4⁺ T cells has no influence (7) or increases IL-9 production (32). To ensure that the mutations causing STAT3 DN LOF activity themselves were not the reason for the mouse and human discrepancy, we cultured naïve CD4 cells from a mouse model of STAT3-mut HIES(29). Similar to the pattern observed with induction of Th9 cells when STAT3 is absent from mouse T-cells, the hypomorphic STAT3mut HIES mice had much higher IL-9 expression compared with WT (Fig 5 A B), Exogenous IL-21 has no obvious effect on IL-9 production, while adding IL-27 –which acts via STAT1 -- significantly diminished IL-9 responses (Fig. 5 A B). With respect to Th9 differentiation, STAT3 therefore plays different roles in humans vs. mice a discrepancy which merits further study.

Discussion

In this study, we find that STAT3LOF and STAT1GOF patients have impaired IL-9 induction ex vivo and Th9 differentiation from naïve cells. Given that IL-21 induction appears to precede IL-9 production in differentiation cultures, and that this too was impaired in these patients, we found that exogenous IL-21 could partially rescue IL-9 production in vitro. The excessive STAT1 signaling leading to T-bet upregulation appears to cause the Th9 defect in patients' cells, as IL-21 mediated induction of SOCS3, or inhibition of STAT1/T-bet could also partially rescue Th9 differentiation. Furthermore, this study also highlights the discrepancy of the role for STAT3 in human vs. mouse Th9 differentiation, as agonist-induced STAT1 activation suppressed Th9 production in mice despite the fact that, in contrast to humans, loss of STAT3 in mice leads to enhanced Th9 production.

Under Th9 differentiation conditions, TGF- β activation of Smad proteins leads to PU.1 upregulation (8, 9), which in turn binds the IL-9 locus altering the epigenetic environments for lineage commitment (33). We previously showed that STAT3 negatively regulates TGF- β signaling via ERBB2-interacting protein (ERBIN), a SMAD anchor for receptor activation and SMAD2/3 binding protein(10), and indeed STAT3LOF PBMC, but not STAT1GOF have enhanced PU.1 induction in the presence of TGF- β . Despite these high levels of PU.1, poor Th9 differentiation in STAT3LOF further highlights the importance of a normal STAT1 signaling in human Th9 development even in the presence of normal PU.1 levels.

Multiple signals are involved in Th9 differentiation. STAT6 (2, 6, 7, 14) not only binds the *IL-9* locus together with STAT5 (34), but also inhibits T-bet (35) and TGF- β -induced FOXP3 expression (6). STAT6 also upregulates several downstream molecules, such as IRF4 and BATF

(36), and we found patients had both normal pSTAT6 signals as well as downstream IRF4 (Fig. S1, Fig. 1E). Our results show that despite the excessive TGF- β signaling, IL-9 levels in STAT3LOF are lower, and that, in conditions of STAT3LOF and STAT1GOF, STAT6 activation alone could not restrain T-bet (Fig. 4B). Another critical factor in Th9 induction is p-STAT5(31), which in our hands (Fig. S3 C) and in previous reports has been reported to be suppressed by transfecting SOCS3 (37). It is therefore highly unlikely that SOCS3 increases IL-9 production in STAT3LOF and STAT1GOF patient cells through STAT5.

IL-21 is used in cancer treatment (38-41). Our finding that IL-21 can partially restore Th9 cells, in vitro, in these rare monogenic diseases points to a set of distinct pathways –impaired STAT3 or enhanced STAT1-- which might help better understand patient responses to such therapy.

Acknowledgments:

We thank Dr. Stuart Tangye for a critical reading of the manuscript.

Author contributions: YZ designed and performed experiments with the help of GS, AMS and AF. JDM supervised experimental design and data analysis. AF and TD recruited human

subjects. YZ and JDM wrote the original draft of the manuscript and all authors reviewed and edited the manuscript.

Figures:

Fig. 1. STAT3LOF and STAT1GOF patient cells have impaired Th9 differentiation.

(A) PBMC from HC, STAT3LOF and STAT1GOF patients were cultured under Th0 or Th9 conditions for 3 days with varied concentrations of TGF- β as indicated. IL-9 and IL-21 levels were detected. (B) Quantification of (A), plus IL-4 levels. (C) Naïve cells from HC, STAT3LOF and STAT1GOF patients were cultured under Th2 or Th9 conditions for 5 days, IL-9, IL-21 and IL-4 expression were detected. (D) Quantification of (C). (E) IL-9, and IRF4 mRNA levels of (C). (F) PU.1 and GATA3 protein and PU.1 mRNA levels in PBMC from HC, STAT3LOF and STAT1GOF. *P < 0.05, **P<0.01, ***P<0.001. ****P<0.001. Data are representative of three independent experiments unless otherwise indicated on graph. Graphs show mean $\pm$ SEM.

Fig. 2. Inhibition p-STAT3 on HC results in reduced IL-21 and IL-9 production, while IL-21 can restore IL-9 levels in STAT3LOF and STAT1GOF patient cells.

(A) Naïve T cells from HC were cultured under Th9 condition with STAT3 antagonist as indicated concentrations. Cell viability, IL-9 and IL-21 levels were detected on day4. (B) IL-9, (C) IL-21 and (D) IL-4 levels of cells in (A) on day2, 3, 4, and 8. * P < 0.05 between 0 μ M

and 0.1 μ M groups. **(E)** Naïve T cells from HC and patients were cultured under Th9 conditions with or without exogenous IL-21 for 5 days. IL-9 and IL-21 expressions were analyzed. **(F)** Quantification of (E). ***P<0.001. Data are representative of three independent experiments unless otherwise indicated on graph. Graphs show mean $\pm$ SEM.

Fig. 3 IL-21 restores IL-9 levels in STAT3LOF and STAT1GOF by inhibiting abnormal p-STAT1 level. **(A)** p-STAT1 levels of naïve cells from HC and STAT3LOF patients cultured under Th0, Th9 or Th9 with exogenous IL-21 conditions for 5 days. **(B)** p-STAT1^{hi} group ratio of naïve cells from HC, STAT3LOF and STAT1GOF patients cultured under condition in (A). **(C)** IL-9 and IL-21 expression of p-STAT1^{low} and p-STAT1^{hi} gating of cells in (A). **(D)** Quantification of IL-9 levels of cells in (B). *P < 0.05, **P<0.01. Data are representative of three independent experiments. Graphs show mean $\pm$ SEM.

Fig. 4 IL-21 restores IL-9 levels in STAT3LOF by increasing SOCS3, and down-regulating T-bet. **(A)** SOCS3 MFI of naïve cells from HC and patients, cultured under Th9 condition with or without IL-21 for 2 days. SOCS3 mRNA levels on 4h. **(B)** T-bet, IRF4 and PU.1 mRNA levels of cells in conditions of (A) for 3days and 24h re-stimulation of anti-CD3. **(C-F)** Naïve cells of HC and patients were transfected with SOCS3-GFP or GFP control plasmid (C D), *STAT1* RNAi, *TBX21* RNAi or scramble RNAi (E F) and then cultured under Th9 condition for 5 days. T-bet expressions on GFP+ gate (C) and IL-9, IL-21 (E) expression was detected, and IL-9, T-bet, IRF4 and PU.1 mRNA levels

on day 3 of culture (D F). **(G)** IL-9 and IL-21 levels of HC naïve cells polarized to Th9 for 5 days, followed transfection with T-bet-GFP or GFP control plasmid. *P < 0.05, **P<0.01, ***P<0.001. ^P<0.05 compared with HC-Sc, ^^P<0.01. #P<0.05 compared with STAT3LOF-Sc, ##P<0.01. &P<0.05 compared with STAT1GOF-Sc, &&P<0.01. Data are representative of three independent experiments. Graphs show mean ± SEM.

Fig. 5 Stat3-mut mice have increased IL-9 production. (A) Naive CD4⁺ T cells from WT and STAT3-mut HIES mice were cultured under Th0 or Th9 condition with IL-21 or IL-27 as indicated. After 5 days, cells were harvested and stimulated with PMA and ionomycin for 5 hours, IL-9 and IL-4 levels on CD4⁺CD44⁺ cells were detected by FACS. **(B)** Quantification of IL-9 levels of cells in (A). **P<0.01. Data are representative of three independent experiments. Graphs show mean ± SEM.

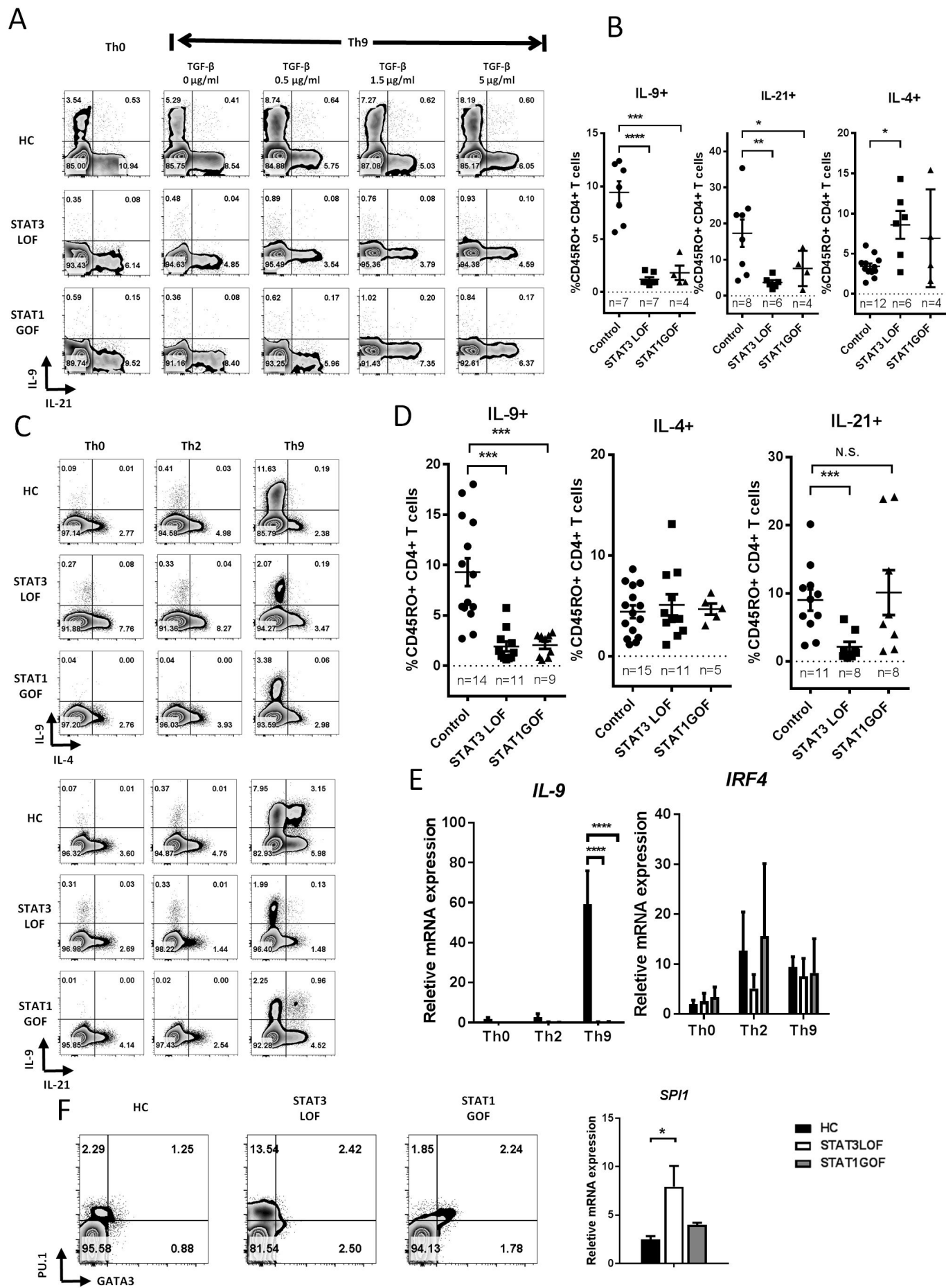
References:

1. Goswami R, Kaplan MH. A brief history of IL-9. *Journal of immunology*. 2011;186(6):3283-8.
2. Staudt V, Bothur E, Klein M, Lingnau K, Reuter S, Grebe N, et al. Interferon-regulatory factor 4 is essential for the developmental program of T helper 9 cells. *Immunity*. 2010;33(2):192-202.
3. Jager A, Dardalhon V, Sobel RA, Bettelli E, Kuchroo VK. Th1, Th17, and Th9 effector cells induce experimental autoimmune encephalomyelitis with different pathological phenotypes. *Journal of immunology*. 2009;183(11):7169-77.
4. Lu Y, Hong S, Li H, Park J, Hong B, Wang L, et al. Th9 cells promote antitumor immune responses in vivo. *The Journal of clinical investigation*. 2012;122(11):4160-71.

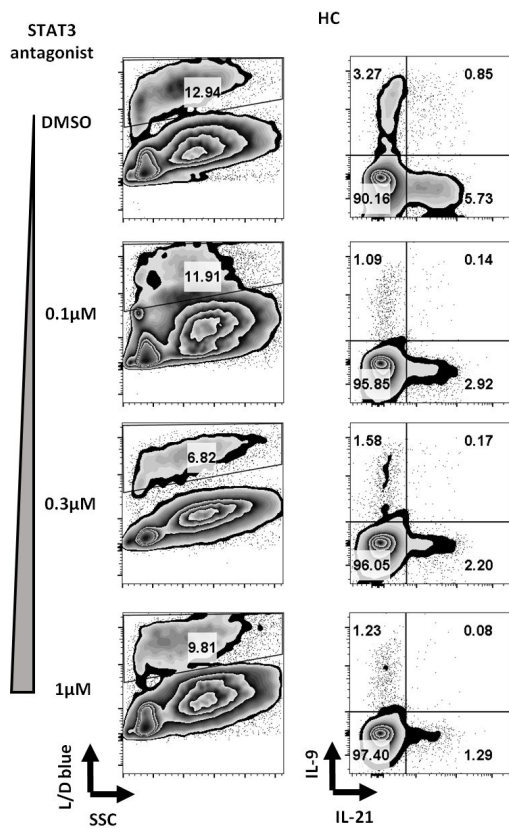
5. Purwar R, Schlapbach C, Xiao S, Kang HS, Elyaman W, Jiang X, et al. Robust tumor immunity to melanoma mediated by interleukin-9-producing T cells. *Nat Med.* 2012;18(8):1248-53.
6. Dardalhon V, Awasthi A, Kwon H, Galileos G, Gao W, Sobel RA, et al. IL-4 inhibits TGF-beta-induced Foxp3⁺ T cells and, together with TGF-beta, generates IL-9⁺ IL-10⁺ Foxp3(-) effector T cells. *Nature immunology.* 2008;9(12):1347-55.
7. Goswami R, Jabeen R, Yagi R, Pham D, Zhu J, Goenka S, et al. STAT6-dependent regulation of Th9 development. *Journal of immunology.* 2012;188(3):968-75.
8. Elyaman W, Bassil R, Bradshaw EM, Orent W, Lahoud Y, Zhu B, et al. Notch receptors and Smad3 signaling cooperate in the induction of interleukin-9-producing T cells. *Immunity.* 2012;36(4):623-34.
9. Chang HC, Sehra S, Goswami R, Yao W, Yu Q, Stritesky GL, et al. The transcription factor PU.1 is required for the development of IL-9-producing T cells and allergic inflammation. *Nature immunology.* 2010;11(6):527-34.
10. Lyons JJ, Liu Y, Ma CA, Yu X, O'Connell MP, Lawrence MG, et al. ERBIN deficiency links STAT3 and TGF-beta pathway defects with atopy in humans. *J Exp Med.* 2017;214(3):669-80.
11. Vegran F, Berger H, Boidot R, Mignot G, Bruchard M, Dosset M, et al. The transcription factor IRF1 dictates the IL-21-dependent anticancer functions of TH9 cells. *Nature immunology.* 2014;15(8):758-66.
12. Liao W, Spolski R, Li P, Du N, West EE, Ren M, et al. Opposing actions of IL-2 and IL-21 on Th9 differentiation correlate with their differential regulation of BCL6 expression. *Proc Natl Acad Sci U S A.* 2014;111(9):3508-13.
13. Ulrich BJ, Verdan FF, McKenzie AN, Kaplan MH, Olson MR. STAT3 Activation Impairs the Stability of Th9 Cells. *Journal of immunology.* 2017;198(6):2302-9.
14. Veldhoen M, Uyttenhove C, van Snick J, Helmby H, Westendorf A, Buer J, et al. Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nature immunology.* 2008;9(12):1341-6.
15. Wong MT, Ye JJ, Alonso MN, Landrigan A, Cheung RK, Engleman E, et al. Regulation of human Th9 differentiation by type I interferons and IL-21. *Immunol Cell Biol.* 2010;88(6):624-31.
16. Becker KL, Rosler B, Wang X, Lachmandas E, Kamsteeg M, Jacobs CW, et al. Th2 and Th9 responses in patients with chronic mucocutaneous candidiasis and hyper-IgE syndrome. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology.* 2016;46(12):1564-74.
17. Yoshimura A, Naka T, Kubo M. SOCS proteins, cytokine signalling and immune regulation. *Nature reviews Immunology.* 2007;7(6):454-65.
18. Chandesris MO, Melki I, Natividad A, Puel A, Fieschi C, Yun L, et al. Autosomal dominant STAT3 deficiency and hyper-IgE syndrome: molecular, cellular, and clinical features from a French national survey. *Medicine (Baltimore).* 2012;91(4):e1-19.
19. Siegel AM, Heimall J, Freeman AF, Hsu AP, Brittain E, Brenchley JM, et al. A critical role for STAT3 transcription factor signaling in the development and maintenance of human T cell memory. *Immunity.* 2011;35(5):806-18.
20. Zhang Y, Ma CA, Lawrence MG, Break TJ, O'Connell MP, Lyons JJ, et al. PD-L1 up-regulation restrains Th17 cell differentiation in STAT3 loss- and STAT1 gain-of-function patients. *J Exp Med.* 2017.

21. Murugaiyan G, Beynon V, Pires Da Cunha A, Joller N, Weiner HL. IFN-gamma limits Th9-mediated autoimmune inflammation through dendritic cell modulation of IL-27. *Journal of immunology*. 2012;189(11):5277-83.
22. Matsuki A, Takatori H, Makita S, Yokota M, Tamachi T, Suto A, et al. T-bet inhibits innate lymphoid cell-mediated eosinophilic airway inflammation by suppressing IL-9 production. *The Journal of allergy and clinical immunology*. 2017;139(4):1355-67 e6.
23. Toubiana J, Okada S, Hiller J, Oleastro M, Lagos Gomez M, Aldave Becerra JC, et al. Heterozygous STAT1 gain-of-function mutations underlie an unexpectedly broad clinical phenotype. *Blood*. 2016;127(25):3154-64.
24. Ozgur TT, Asal GT, Gurgey A, Tezcan I, Ersoy F, Sanal O. Acquired factor VIII deficiency associated with a novel primary immunodeficiency suggestive of autosomal recessive hyper IgE syndrome. *Journal of pediatric hematology/oncology*. 2007;29(5):327-9.
25. Gorin LJ, Jeha SC, Sullivan MP, Rosenblatt HM, Shearer WT. Burkitt's lymphoma developing in a 7-year-old boy with hyper-IgE syndrome. *The Journal of allergy and clinical immunology*. 1989;83(1):5-10.
26. Leonard GD, Posadas E, Herrmann PC, Anderson VL, Jaffe ES, Holland SM, et al. Non-Hodgkin's lymphoma in Job's syndrome: a case report and literature review. *Leukemia & lymphoma*. 2004;45(12):2521-5.
27. Boos AC, Hagl B, Schlesinger A, Halm BE, Ballenberger N, Pinarci M, et al. Atopic dermatitis, STAT3- and DOCK8-hyper-IgE syndromes differ in IgE-based sensitization pattern. *Allergy*. 2014;69(7):943-53.
28. Hox V, O'Connell MP, Lyons JJ, Sackstein P, Dimaggio T, Jones N, et al. Diminution of signal transducer and activator of transcription 3 signaling inhibits vascular permeability and anaphylaxis. *The Journal of allergy and clinical immunology*. 2016;138(1):187-99.
29. Steward-Tharp SM, Laurence A, Kanno Y, Kotlyar A, Villarino AV, Sciume G, et al. A mouse model of HIES reveals pro- and anti-inflammatory functions of STAT3. *Blood*. 2014;123(19):2978-87.
30. Milner JD, Holland SM. The cup runneth over: lessons from the ever-expanding pool of primary immunodeficiency diseases. *Nature reviews Immunology*. 2013;13(9):635-48.
31. Schmitt E, Germann T, Goedert S, Hoehn P, Huels C, Koelsch S, et al. IL-9 production of naive CD4⁺ T cells depends on IL-2, is synergistically enhanced by a combination of TGF-beta and IL-4, and is inhibited by IFN-gamma. *Journal of immunology*. 1994;153(9):3989-96.
32. Olson MR, Verdant FF, Hufford MM, Dent AL, Kaplan MH. STAT3 Impairs STAT5 Activation in the Development of IL-9-Secreting T Cells. *Journal of immunology*. 2016;196(8):3297-304.
33. Goswami R, Kaplan MH. Gcn5 is required for PU.1-dependent IL-9 induction in Th9 cells. *Journal of immunology*. 2012;189(6):3026-33.
34. Yang XO, Zhang H, Kim BS, Niu X, Peng J, Chen Y, et al. The signaling suppressor CIS controls proallergic T cell development and allergic airway inflammation. *Nature immunology*. 2013;14(7):732-40.
35. Kaplan MH, Hufford MM, Olson MR. The development and in vivo function of T helper 9 cells. *Nature reviews Immunology*. 2015;15(5):295-307.
36. Jabeen R, Goswami R, Awe O, Kulkarni A, Nguyen ET, Attenasio A, et al. Th9 cell development requires a BATF-regulated transcriptional network. *The Journal of clinical investigation*. 2013;123(11):4641-53.

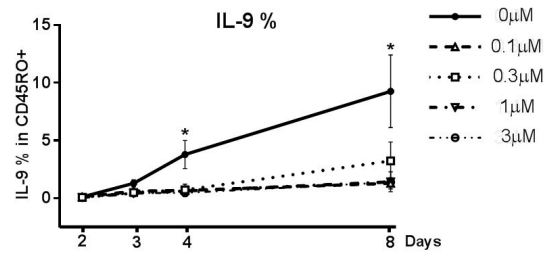
37. Barclay JL, Anderson ST, Waters MJ, Curlewis JD. SOCS3 as a tumor suppressor in breast cancer cells, and its regulation by PRL. *Int J Cancer*. 2009;124(8):1756-66.
38. Thompson JA, Curti BD, Redman BG, Bhatia S, Weber JS, Agarwala SS, et al. Phase I study of recombinant interleukin-21 in patients with metastatic melanoma and renal cell carcinoma. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 2008;26(12):2034-9.
39. Davis ID, Brady B, Kefford RF, Millward M, Cebon J, Skrumsager BK, et al. Clinical and biological efficacy of recombinant human interleukin-21 in patients with stage IV malignant melanoma without prior treatment: a phase IIa trial. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2009;15(6):2123-9.
40. Santegoets SJ, Turksma AW, Powell Jr DJ, Hooijberg E, de Gruijl TD. IL-21 in cancer immunotherapy: At the right place at the right time. *Oncoimmunology*. 2013;2(6):e24522.
41. Spolski R, Leonard WJ. Interleukin-21: a double-edged sword with therapeutic potential. *Nature reviews Drug discovery*. 2014;13(5):379-95.



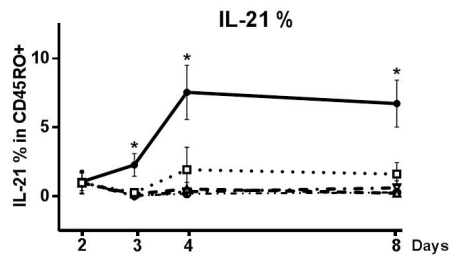
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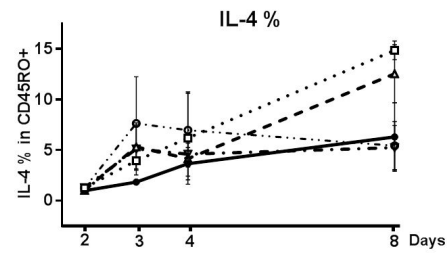
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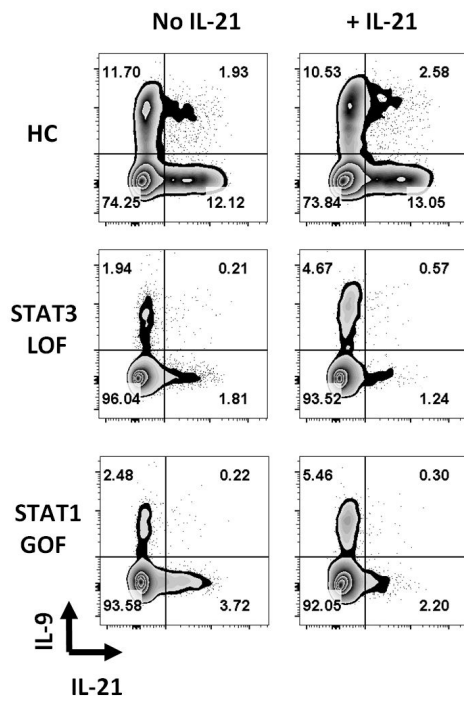
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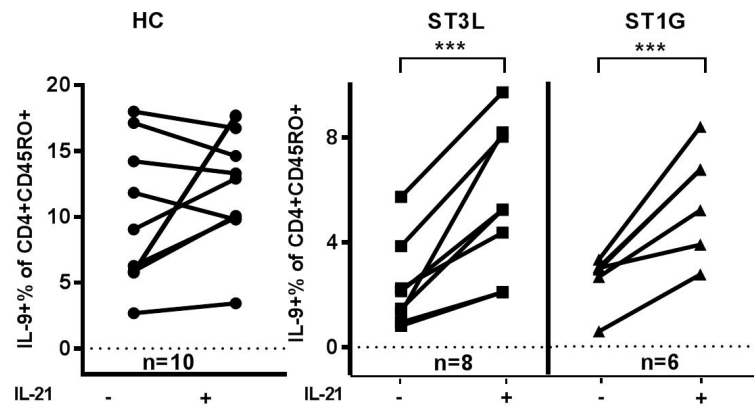
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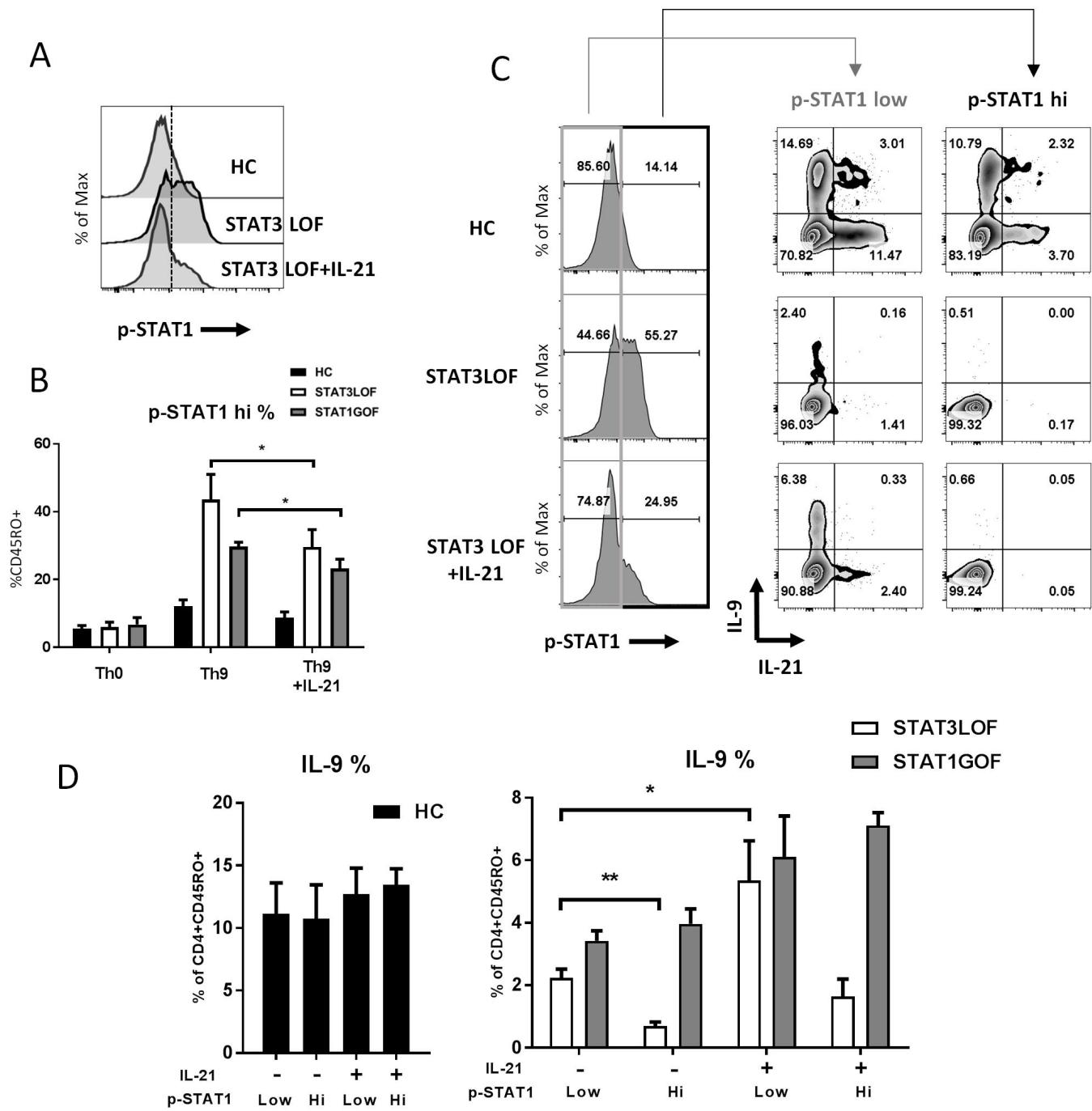


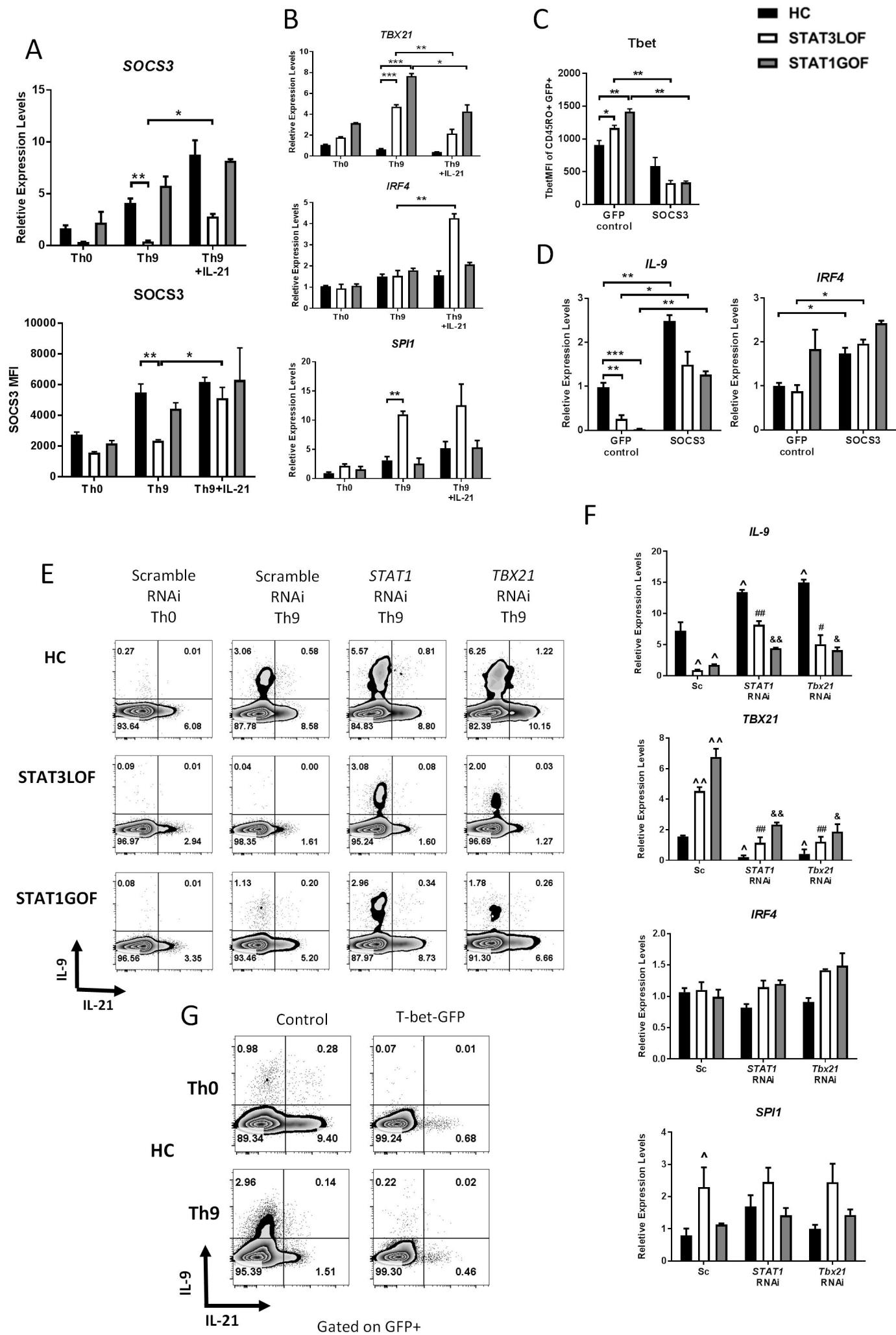
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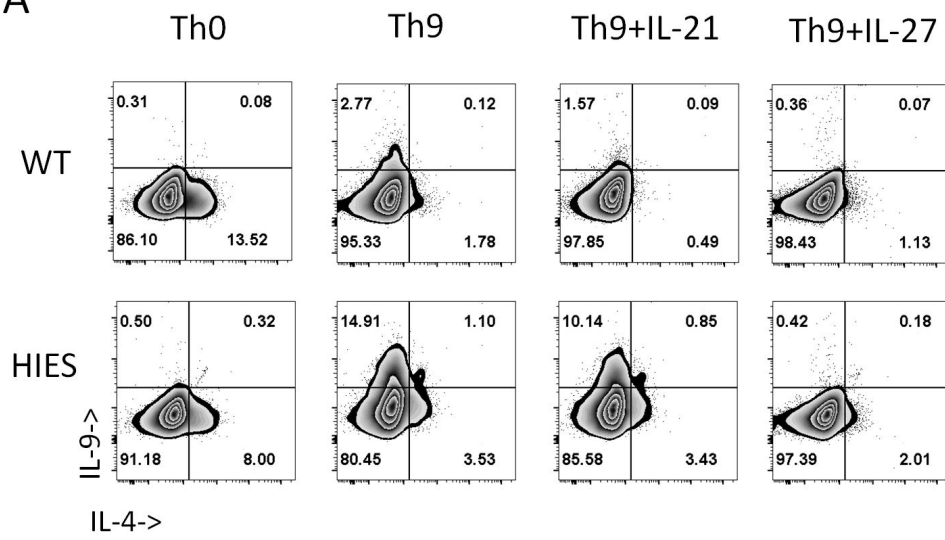
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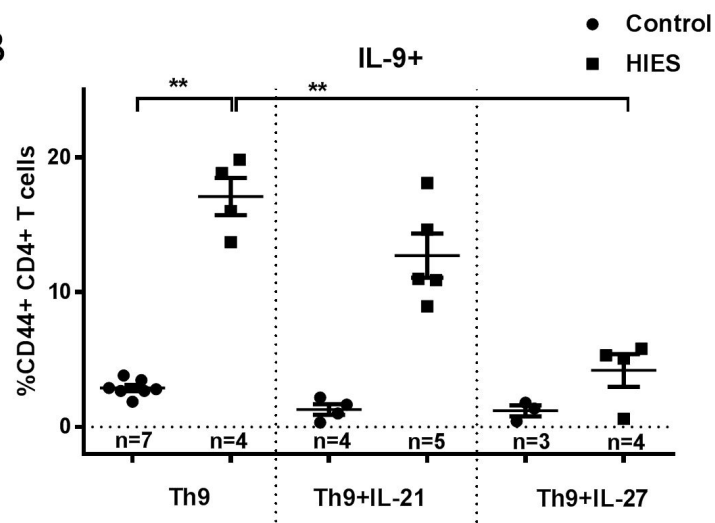




A



B



Supplementary Fig. 1 STAT3 LOF and STAT1 GOF cells have normal pSTAT6. (A)

pSTAT6 in HC and patients PBMCs after stimulation with IL-4 for 30 minutes. **(B)**

GATA3 levels of Th9 culture from naïve cells of HC and patients.

Supplementary Fig. 2 Cytokines and antibody affects HC and patients Th9 differentiation.

(A) Naïve cells from HC, STAT3LOF and STAT1GOF patients were cultured under Th9 conditions for 5 days with cytokines or antibodies indicated. **(B)** IL-9 relative levels change by adding variety of cytokines. **(C)** PU.1 and GATA3 levels on PBMC from HC and patients stimulated under Th9 with or without IL-21 for 4 hours. **(D)** IL-4 levels change by adding IL-21 on Naïve cells under Th9 polarization. Data are representative of three independent experiments.

Supplementary Fig. 3 Over expression SOCS3 partially restores IL-9. (A) Naïve cells of HC

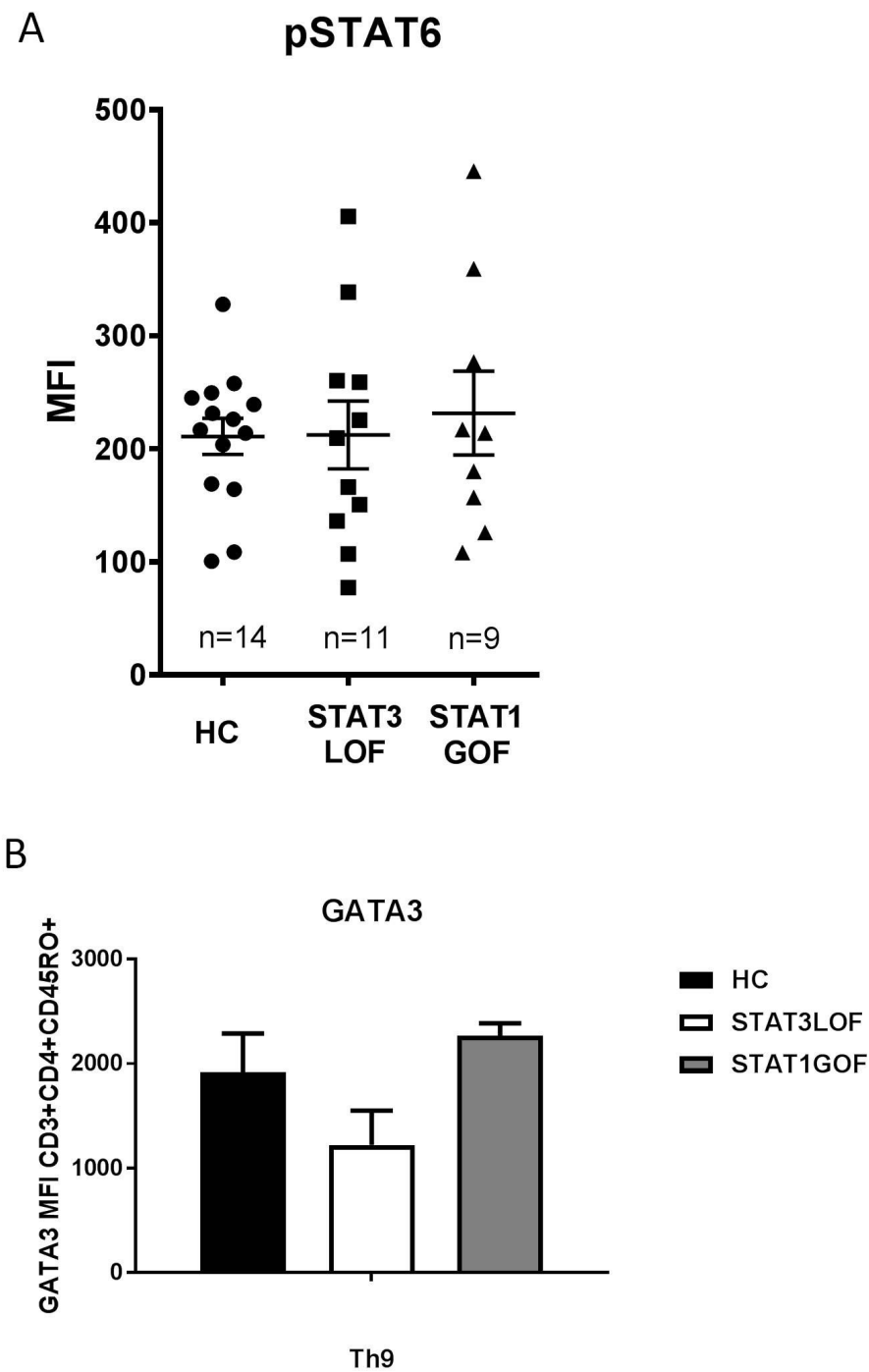
and patients were transfected with SOCS3-GFP or GFP control plasmid and cultured under Th9 conditions for 5 days. IL-9 and IL-21 levels were detected on harvest day. **(B)** Quantification of IL-9 levels of cells in (A). **(C)** 24h hours after transfection, cells were stimulated by adding IL-2, and 30m later, p-STAT5 levels on GFP+ groups were analyzed. *P<0.05. Data are representative of three independent experiments.

Table S1. Genotype and phenotype information on the patients in the study. Naïve cells from patients were cultured under Th9 conditions for 5 days, and IL-9% was detected.

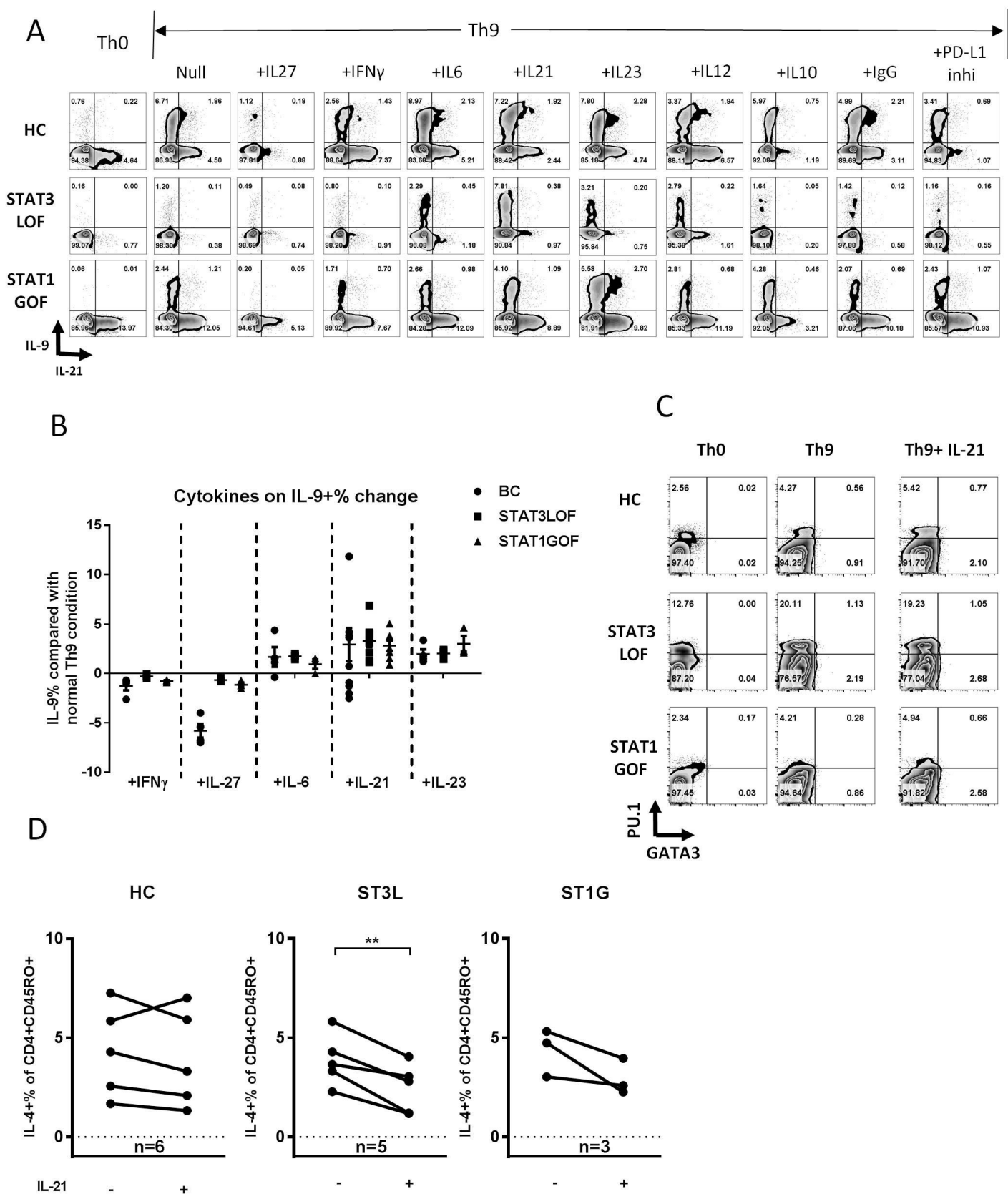
Age/sex	Disease Mutation Domain	CMC?	Other infectious history	Other clinical features	IL-9 level %
36/F	STAT3 LOF 1144C-T; R382W; DNA Binding	yes	Sino-oto-pulmonary infections with bronchiectasis, SSTI, UTI with urosepsis, recurrent HSV	large liver hemangioma requiring resection, with persistent biliary leak post-operatively	0.78
13/F	STAT3 LOF 1144 C-T; R382W; DNA Binding	no	SSTI, sinus/ ear infections	severe scoliosis requiring rod placement	2.62
32/M	STAT3 LOF 1144 C-T; R382W; DNA Binding	yes	Sino-oto-pulmonary infections with bronchiectasis, SSTI	Non-Hodgkin's lymphoma, Cardiomyopathy	0.73
18/M	STAT3 LOF 1027 G-T; V343F; DNA Binding	yes	Sino-oto-pulmonary infections with bronchiectasis, SSTI	severe scoliosis requiring rod placement	1.31
42/F	STAT3 LOF 1909 G-A; V637M; SH2	yes	Sino-oto-pulmonary infections with bronchiectasis, SSTI	laryngeal abscess leading to tracheostomy; severe scoliosis leading to rod placement	0.62
11/F	STAT3 LOF 1144 C-T; R382W; DNA Binding	no	Sino-oto-pulmonary infections, SSTI	Recto-vaginal fistulae s/p correction	0.95
46/F	STAT3 LOF 1151 T-C; F384S; DNA Binding	yes	Sino-oto-pulmonary infections with bronchiectasis, SSTI		1.47
33/F	STAT3 LOF	yes	Sino-oto-pulmonary	hypothyroid	

	1144 C-T; R382W; DNA Binding		infections with bronchiectasis, SSTI		2.25
10/M	STAT3 LOF 1309 C-T; H436Y; DNA Binding	no	Sino-oto-pulmonary infections, SSTI		5.73
44/F	STAT3 LOF 1909 G-A; V637M; SH2	yes	Sino-oto-pulmonary infections with bronchiectasis, SSTI		0.82
19/F	STAT3 LOF 1268 G-A; R423Q; DNA Binding	no	Sinus		3.85

Supplemental Fig. 1



Supplemental Fig. 2



Supplemental Fig. 3

