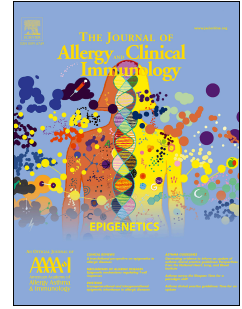


Accepted Manuscript

Hedgehog signalling promotes Th2 differentiation in naïve human CD4 T cells

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PII: S0091-6749(19)30948-0

DOI: <https://doi.org/10.1016/j.jaci.2019.07.011>

Reference: YMAI 14096

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

Received Date: 5 April 2019

Revised Date: 2 July 2019

Accepted Date: 15 July 2019

Please cite this article as: Yáñez DC, Lau C-I, Chawda MM, Ross S, Furmanski AL, Crompton T, Hedgehog signalling promotes Th2 differentiation in naïve human CD4 T cells, *Journal of Allergy and Clinical Immunology* (2019), doi: <https://doi.org/10.1016/j.jaci.2019.07.011>.

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1 **Hedgehog signalling promotes Th2 differentiation in naïve human CD4 T**
2 **cells**

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21 The authors report no conflicts of interest.

22

23 This research was funded by grants from MRC, Wellcome Trust, Great Ormond

24 Street Children's Charity and an investigator-initiated grant from Pfizer. DY received

25 a fellowship from SENESCYT and AF from AsthmaUK. Research at UCL Great

26 Ormond Street Institute of Child Health is supported by the NIHR BRC at Great
27 Ormond Street Hospital.

28

29 **Capsule summary:** Here we show that differentiation of human naïve CD4 T-cells to
30 Th2 is promoted by Hedgehog signaling and attenuated by SMO-inhibition. As
31 Hedgehog proteins are produced by epithelial tissues this finding is important to
32 understanding atopic disease.

33

34 **Key words:** Hh signalling, SHH, Th2 differentiation, SMO

35 **Abbreviations:**

36 FACS: Fluorescence-activated cell sorting

37 GLI: Glioma-associated Oncogene

38 Hh: Hedgehog

39 IFN- γ : Interferon gamma

40 PTCH1: PATCHED1

41 r-Shh: recombinant Sonic hedgehog

42 SHH: Sonic Hedgehog Homologue

43 SMO: SMOOTHENED

44

45 **To the Editor:**

46 Hedgehog (Hh) proteins are inter-cellular signalling molecules that control
47 development and tissue homeostasis. They also regulate thymocyte development and
48 peripheral T-cell activation in mouse and human, and have recently been shown to
49 promote Th2 differentiation and function in mice (1-4). SHH is involved in
50 homeostasis of many epithelial tissues, and as these tissues are the sites of allergic
51 disease, it is important to understand how Hh signalling influences human CD4 T
52 helper differentiation. Here we show that Hedgehog signalling promotes human Th2
53 differentiation, using materials and methods described in (3, 5) and the
54 Supplementary File.

55 We used quantitative(Q) RT-PCR to evaluate gene expression of components of the
56 Hh signaling pathway in naive human CD4 T-cells stimulated for 48h in Th0, Th1 or
57 Th2 polarizing conditions. Expression of the Hh-responsive transcription factors *GLI1*
58 and *GLI2* and the Hh cell surface receptor *PTCH1* were higher in CD4 T-cells
59 cultured under Th2 skewing conditions, compared to Th0 or Th1 conditions (Fig.1A),
60 suggesting that Hh signaling is involved in human Th differentiation or function. As
61 *GLI1* and *PTCH1* are Hh-target genes, their higher expression in Th2-differentiated
62 cells indicates that this population has overall higher Hh-mediated transcription.

63 To test the influence of SHH signaling on Th differentiation, we stimulated purified
64 naïve human CD4 T-cells from 12 independent randomly selected anonymous donors
65 for 4 days under skewing conditions, with or without a single dose of recombinant
66 Shh (rShh). Treatment with rShh significantly enhanced expression of the Th2-
67 transcription factor GATA3 in cells stimulated under Th2 conditions, whereas
68 GATA3 expression in Th0 conditions, and TBET expression in Th0 or Th1 conditions
69 were not affected (Fig.1B-C). Treatment of Th2-skewing cultures with rShh also

70 increased the concentration of IL-4 in supernatants after 4 days of culture, compared
71 to control Th2-skewing cultures (Fig.1D). Interestingly, the concentration of IFN- γ
72 was lower when rShh was added compared to control Th1 cultures (Fig.1E). After 7
73 days of culture and CD3/CD28 restimulation, the proportion of CD4 T-cells that
74 expressed IL-4 was significantly increased in the presence of rShh under Th2
75 conditions (Fig. 1F). In contrast, the percentage of cells that expressed IFN- γ was
76 reduced in Th1+rShh cultures compared to Th1 cells (Fig. 1G). Shh-treatment
77 increased *GATA3* and *IL4* expression in Th2 cultures (Fig.1H), whereas rShh-
78 treatment decreased *IFNG* and *TBX21 (TBET)* expression in Th1 cultures (Fig.1I).
79 These data indicate that Hh signalling promotes Th2 differentiation in human CD4 T-
80 cells, with simultaneous repression of IFN- γ and TBET.

81 We then investigated whether pharmacological inhibition of the Hh signalling
82 pathway, by treatment with an inhibitor of the non-redundant Hh-signal transduction
83 molecule SMO (PF-04449913) would impair Th2 differentiation(6). The proportion
84 of cells that expressed the Th1 lineage-specific transcription factor TBET was not
85 affected by SMO-inhibitor treatment under skewing conditions (Fig.2A). Likewise,
86 no differences were found in the expression of GATA3 under neutral or Th1
87 conditions (Fig.2B). However, SMO-inhibitor-treatment significantly reduced the
88 proportion of CD4 T-cells that expressed GATA3 and KI67 (marker of proliferation)
89 when cultured under Th2 skewing conditions (Fig.2B-C). SMO-inhibition did not
90 affect the percentage of cells that expressed IFN- γ in Th1 conditions, and as expected
91 IL-4 expression was low in Th1 conditions in both control and SMO-inhibitor-treated
92 cultures (Fig.2D). When cultured under Th2- conditions, however, the percentage of
93 cells that expressed IL-4 was significantly reduced by SMO-inhibitor-treatment
94 (Fig.2E). Analysis of cytokine concentration in culture supernatants by ELISA

95 showed that IFN- γ levels were similar in both groups under Th1 conditions (Fig. 2F),
96 but under Th2 conditions significantly lower concentrations of IL-4 were found in the
97 SMO-inhibitor group compared to controls (Fig. 2G). Finally, we investigated the
98 transcript levels of *IL4* and *IFNG* by QRT-PCR. In Th2 skewed cells *IL4* expression
99 was significantly lower in SMO-inhibitor-treated cultures than controls (Fig. 2H),
100 whereas *IFNG* transcript levels were not different between groups under Th1
101 conditions (Fig. 2I). Taken together, these analyses indicated that attenuation of Hh
102 signal transduction by treatment with the SMO-inhibitor reduced Th2 differentiation,
103 but did not affect Th1 fate.

104 Here we show that Hedgehog signalling promotes Th2 differentiation in human CD4
105 T-cells. We found that treatment of naïve CD4 T-cells with rShh under Th2 skewing
106 conditions increased the expression of the transcription factor GATA3, a reliable
107 indicator of Th2 transcriptional identity. In support of this, *IL4* expression was
108 enhanced and IL-4 cytokine production was increased in Th2 cultures on treatment
109 with rShh. In contrast, rShh treatment antagonised Th1 differentiation in Th1 cultures,
110 leading to lower *IFNG* and *TBX21* expression and a lower proportion of cells
111 expressing intracellular IFN- γ . Attenuation of Hh signal transduction by
112 pharmacological SMO-inhibition reduced Th2 differentiation: both GATA3 and IL4
113 expression were significantly decreased.

114 In murine T helper differentiation, Hh signalling promotes Th2 differentiation,
115 skewing the overall pattern of transcription to a Th2-like profile, and *Il4* is a Gli2
116 target gene in murine T-cells(3). Importantly, Hh pathway activation in T-cells has
117 physiological relevance in a murine model of allergic asthma, as by favouring Th2
118 polarization and cytokine production, it contributes to disease severity (3, 7).

119 In humans, GWAS linked components of the Hedgehog (Hh) signalling pathway to
120 allergic asthma (8), and a recent study found that children with asthma presented with
121 higher levels of SHH in airway epithelia than healthy controls (9).

122 Here we provide in vitro evidence that Hedgehog signalling enhances Th2
123 differentiation in human CD4 T-cells. One strength of our study is that our
124 experiments were performed using cells isolated from 12 different unknown leucocyte
125 cone donors, and we obtained consistent experimental results from all donors,
126 independent of their age or gender (of which we had no knowledge). A weakness of
127 our study is that it was limited to in vitro experimentation. In the future it will
128 therefore be interesting to assess the Th differentiation status of T-cell populations
129 isolated from patient samples from sufferers of asthma to obtain further ex vivo
130 evidence that Hh signalling is involved in human Th2 responses. This will be
131 important to our understanding of human atopic disease such as asthma, in which Th2
132 T-cell responses drive disease.

133

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177 **Figure Legend**178 **Figure 1. Shh treatment increases Th2 differentiation in vitro**

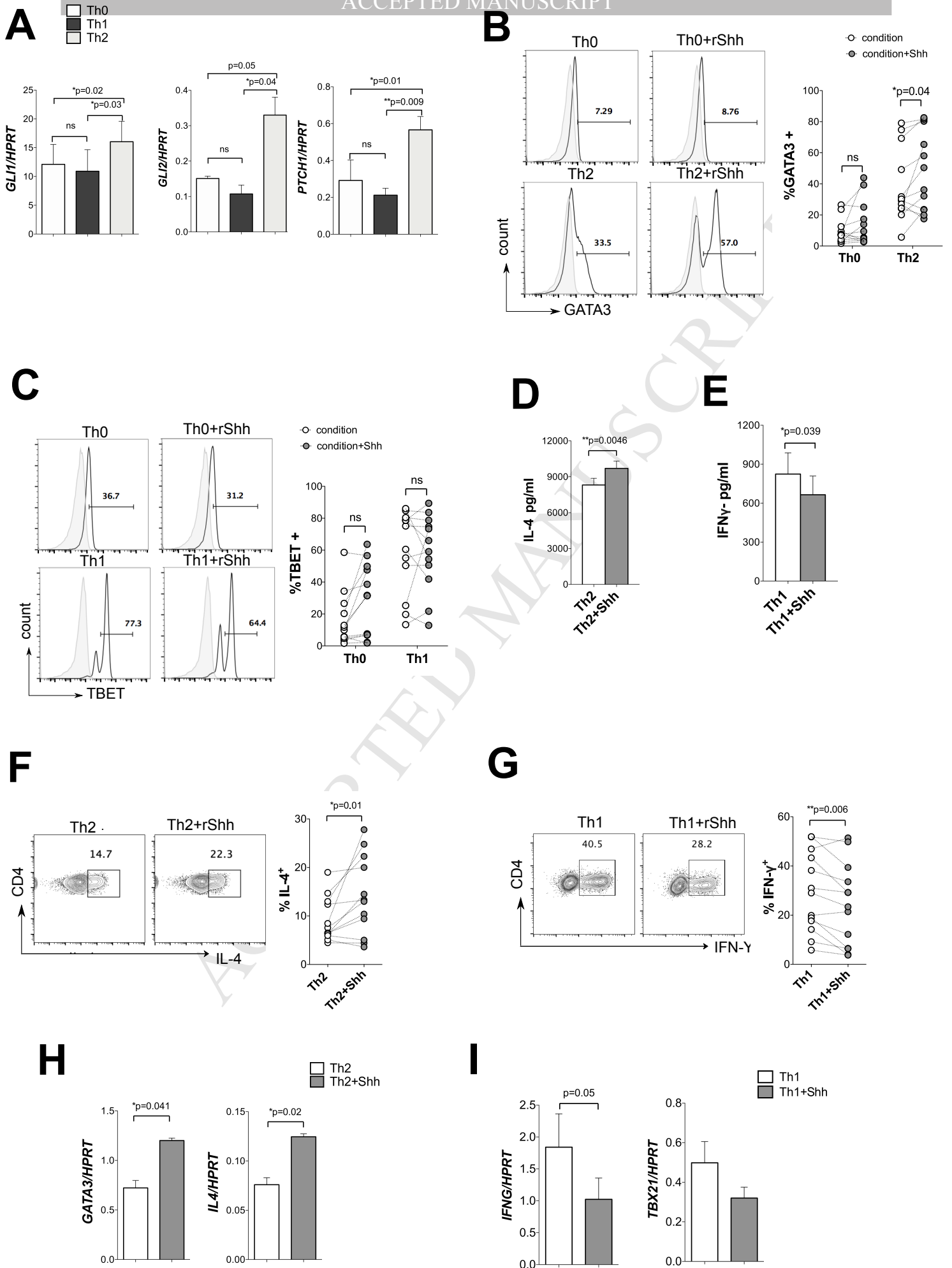
179 Naïve CD4 T-cells (n=12 donors) stimulated under Th-skewing conditions, +/-rShh
180 **(B-I)**; analysed at 48h **(A)**, day4 **(B-E)** and day7+restimulation **(F-I)**. Plots:
181 mean±SEM; each point represents an individual donor. **(A)** Gene expression (QRT-
182 PCR) (n=3). **(B-C)** FACS-histograms show intracellular-expression (gated on CD4+)
183 of **(B)** GATA3; **(C)** TBET. Grey-overlays: control stain. Scatter-plots: percentage of
184 positive cells. **(D-E)** Cytokine concentration (ELISA) in supernatants. **(F-G)** FACS-
185 plots: CD4 and intracellular-cytokine expression. Scatter-plots: percentage cytokine-
186 positive. **(H-I)** Gene expression (QRT-PCR, n=3). Paired 2-tailed t-test; *p<0.05; **
187 p<0.01.

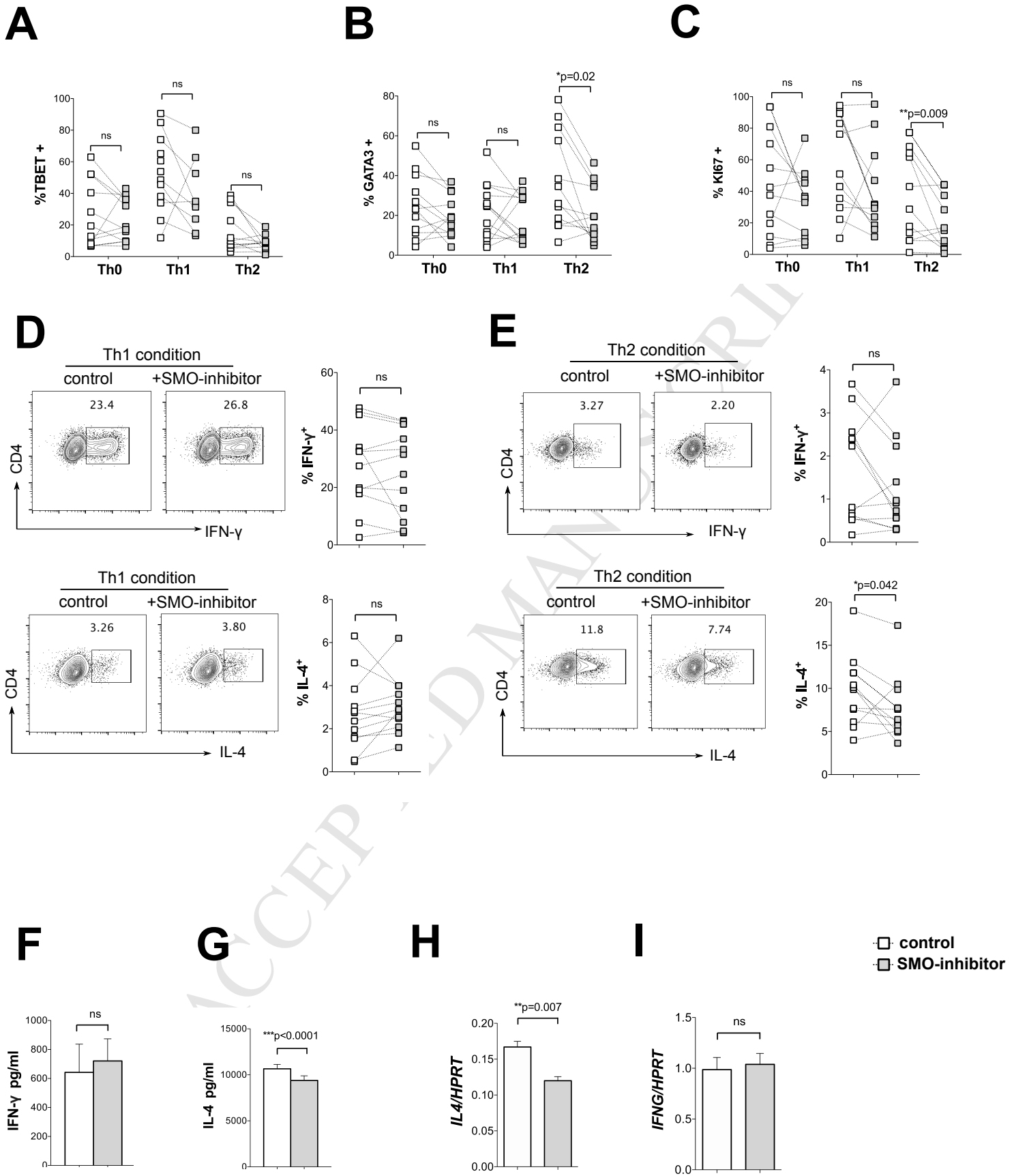
188 **Figure 2. SMO-inhibition decreases Th2 differentiation in vitro.**

189 Naïve CD4 T-cells (n=12 donors) stimulated under Th-skewing conditions with
190 SMO-inhibitor (grey-fill) or DMSO (control; open bars/squares), on day4 **(A-C, F-G)**
191 and day7+restimulation **(D-E, H-I)**. Scatter-plots: mean±SEM; each point represents
192 an individual donor.
193 **(A-C)** Percentage of CD4+ cells that were positive for intracellular staining against
194 **(A)** TBET **(B)** GATA3 and **(C)** KI67. **(D-E)** FACS-plots show expression of CD4
195 and intracellular IFN- γ (upper-plots) or intracellular IL-4 (lower-plots) in cells
196 cultured in Th1**(D)** or Th2**(E)** conditions. Scatter plots show the percentage of CD4+
197 cells that stained positive with the stated cytokine. **(F-G)** Cytokine concentration
198 (ELISA) in supernatants from Th1**(F)** and Th2**(G)** cultures. **(H-I)** Gene expression
199 (QRT-PCR) in cells from Th2**(H)** and Th1**(I)** cultures (3 random donors). Paired 2-
200 tailed t-test; *p<0.05; ** p<0.01; ***p<0.001.

Figure 1

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Material and Methods

Human naïve CD4 purification and culture

Human peripheral blood mononuclear cells (PBMCs) were freshly isolated from randomly-selected unknown leucocyte-cone donors (UK NHS Blood and Transplant Centre) by gradient centrifugation using Lymphoprep (Axis-Shield; Oslo, Norway). The donors to the UK NHS Blood and Transplant Centre are aged between 17 and 65 years old and we had no knowledge of their age, gender or identity. Ethical approval was authorized by local area NHS REC (research ethics committee). Naïve CD4 T-cells ($CD3^+CD4^+CD45RA^+CD45RO^-$) were magnetic-bead purified from PBMC using EasySep Isolation Kit (StemCell). Purity of naïve CD4 T-cells was analysed by flow cytometry and exceeded 95%. After magnetic-bead isolation, naïve CD4 T-cells were rested for 3-5 hours and then plated in 96 round well plates at 1×10^6 cells/ml. Cells were stimulated in complete RPMI (supplemented with 10% FBS, 1% penicillin-streptomycin and 10^{-5} M 2-mercaptoethanol) with 5 µg/ml plate-bound anti-CD3 antibody (clone UCHT1) and anti-CD28 antibody (eBioscience). For Th0 conditions no cytokines were added; For Th1: anti-IL-4 (5 µg/ml), rIL-12 (20 ng/ml), rIFN- γ (10 ng/ml); and for Th2: anti-IFN γ (2.5 µg/ml), rIL-4 (20 ng/ml) were added. After 4 days, cells were expanded in human rIL-2 (100 U/mL) for 3 days in fresh medium containing the same skewing cytokines and neutralising antibodies but in the absence of anti-CD3 and CD28 stimulation. Cells were then restimulated for 16 hours by addition of soluble anti-CD3 and anti-CD28 (1 µg/ml) prior to gene expression and cytokine analysis. Where stated, recombinant Shh (rShh) (R&D Systems) was added at a final concentration of 0.5 µg/ml at the initiation of culture, and again on day 4 when the medium was changed and the cells expanded by addition of rIL2.

Where stated, SMO-inhibitor (PF-04449913; Pfizer, dissolved in DMSO) was added to cultures for a final concentration of 1nM/ml; (1 μ l/ml) and an equivalent volume/concentration of DMSO alone was added to control wells (DMSO at 1:10,000 final dilution). This treatment or control was added to the corresponding wells every day until the end of the experiment.

For intracellular (ic) cytokine staining, CD4 T-cells were stimulated for 4 h with 50 ng/ml PMA (Sigma-Aldrich), 500ng/ml ionomycin (Sigma- Aldrich), and 3 μ g/ml Brefeldin A (eBioscience).

Flow cytometry

Cells were stained using combinations of directly conjugated antibodies from Thermo Fisher or Biolegend in FACS buffer (5% Fetal Bovine serum and 0.01% sodium azide in 1X PBS), acquired on a C6 Accuri flow cytometer (BD Biosciences) and analyzed using FlowJo v10.6. For intracellular staining, CD4 T-cells were stained with anti-CD4 for cell-surface staining and then incubated with Fixation/Permeabilization solution (eBioscience) for 20 minutes in the dark. Following this, cells were washed twice with permeabilization buffer then stained with specific antibodies in permeabilization buffer for 40 minutes. After incubation, cells were washed with permeabilization buffer and resuspended in FACS buffer for FACS analysis.

ELISA

IFN- γ and IL-4 cytokines were measured using Ready-Set-Go! Kits (eBioscience), according to the manufacturer's instructions.

Quantitative (Q)RT-PCR

RNA was extracted using PicoPure kit (Applied Biosystems). cDNA was synthesized using High Capacity cDNA reverse transcription kit (Applied Biosystems) and were analysed on an iCycler (Bio-Rad Laboratories, Hercules, CA) using SYBR Green Supermix (Bio-Rad) following manufacturer's guidelines. RNA levels obtained from each sample were measured relative to the housekeeping gene *HPRT*. All primers were purchased from Qiagen.

Statistical analysis

Paired two-tailed student's *t* test was used for statistical analysis for comparison of in vitro treatments of cells from a given individual. Probabilities were considered significant if $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).