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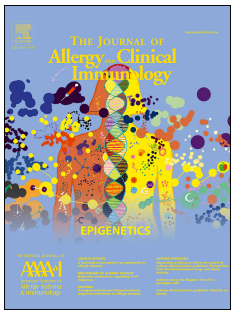

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Hedgehog signalling promotes Th2 differentiation in naïve human CD4 T cells

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

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

Abbreviations:

FACS: Fluorescence-activated cell sorting

GLI: Glioma-associated Oncogene

Hh: Hedgehog

IFN-γ: Interferon gamma

PTCH1: PATCHED1

r-Shh: recombinant Sonic hedgehog

SHH: Sonic Hedgehog Homologue

SMO: SMOOTHENED

To the Editor:

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

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

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

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

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

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

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

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

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

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

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Figure Legend

Figure 1. Shh treatment increases Th2 differentiation in vitro

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

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

Naïve CD4 T-cells (n=12 donors) stimulated under Th-skewing conditions with SMO-inhibitor (grey-fill) or DMSO (control; open bars/squares), on day4 (A-C, F-G) and day7+restimulation (D-E, H-I). Scatter-plots: mean±SEM; each point represents an individual donor.

(A-C) Percentage of CD4+ cells that were positive for intracellular staining against (**A**) TBET (**B**) GATA3 and (**C**) KI67. (**D-E**) FACS-plots show expression of CD4 and intracellular IFN-γ (upper-plots) or intracellular IL-4 (lower-plots) in cells cultured in Th1(**D**) or Th2(**E**) conditions. Scatter plots show the percentage of CD4+ cells that stained positive with the stated cytokine. (**F-G**) Cytokine concentration (ELISA) in supernatants from Th1(**F**) and Th2(**G**) cultures. (**H-I**) Gene expression (QRT-PCR) in cells from Th2(**H**) and Th1(**I**) cultures (3 random donors). Paired 2-tailed t-test; *p<0.05; ** p<0.01; ***p<0.001.

Figure 1

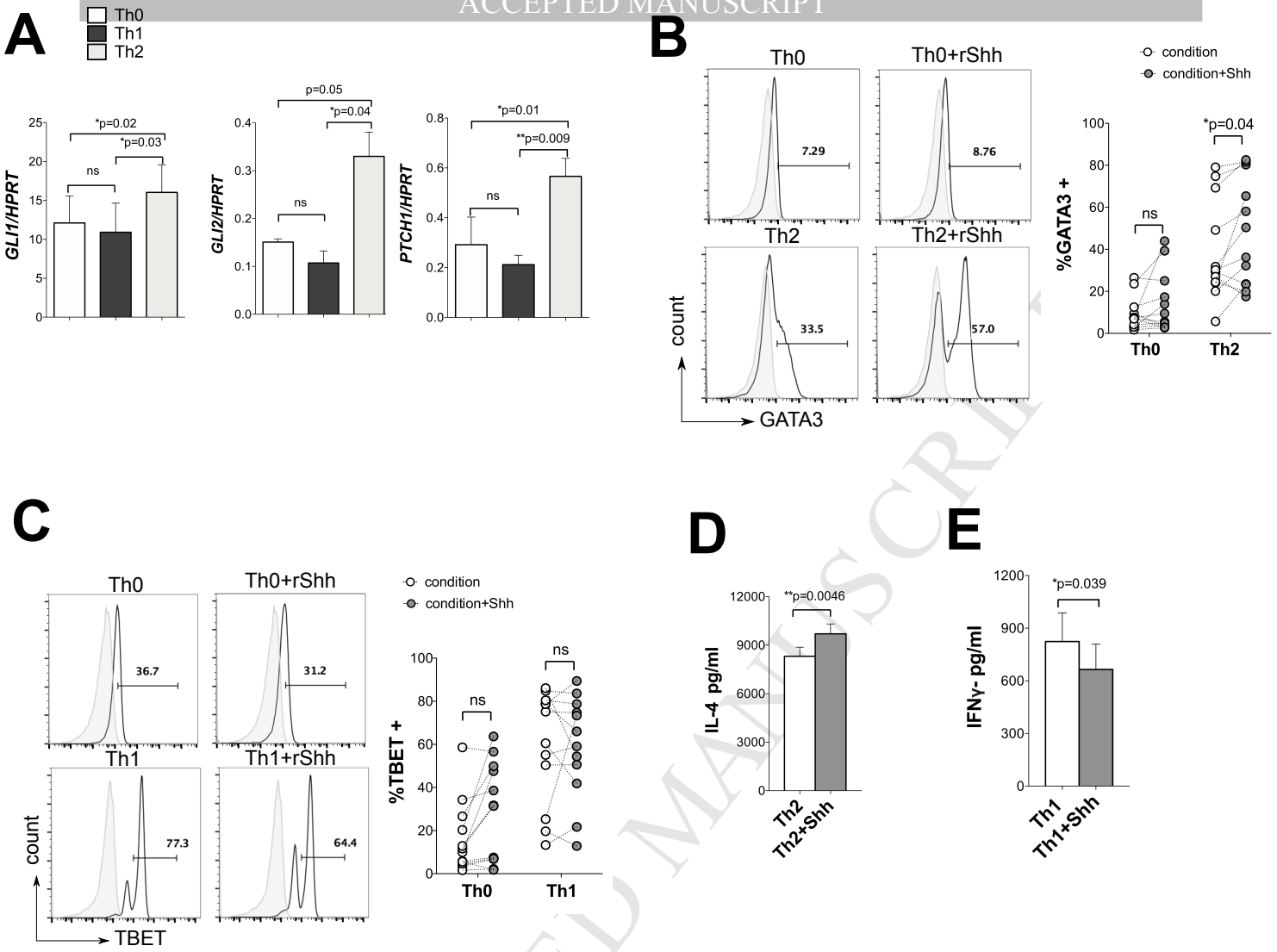
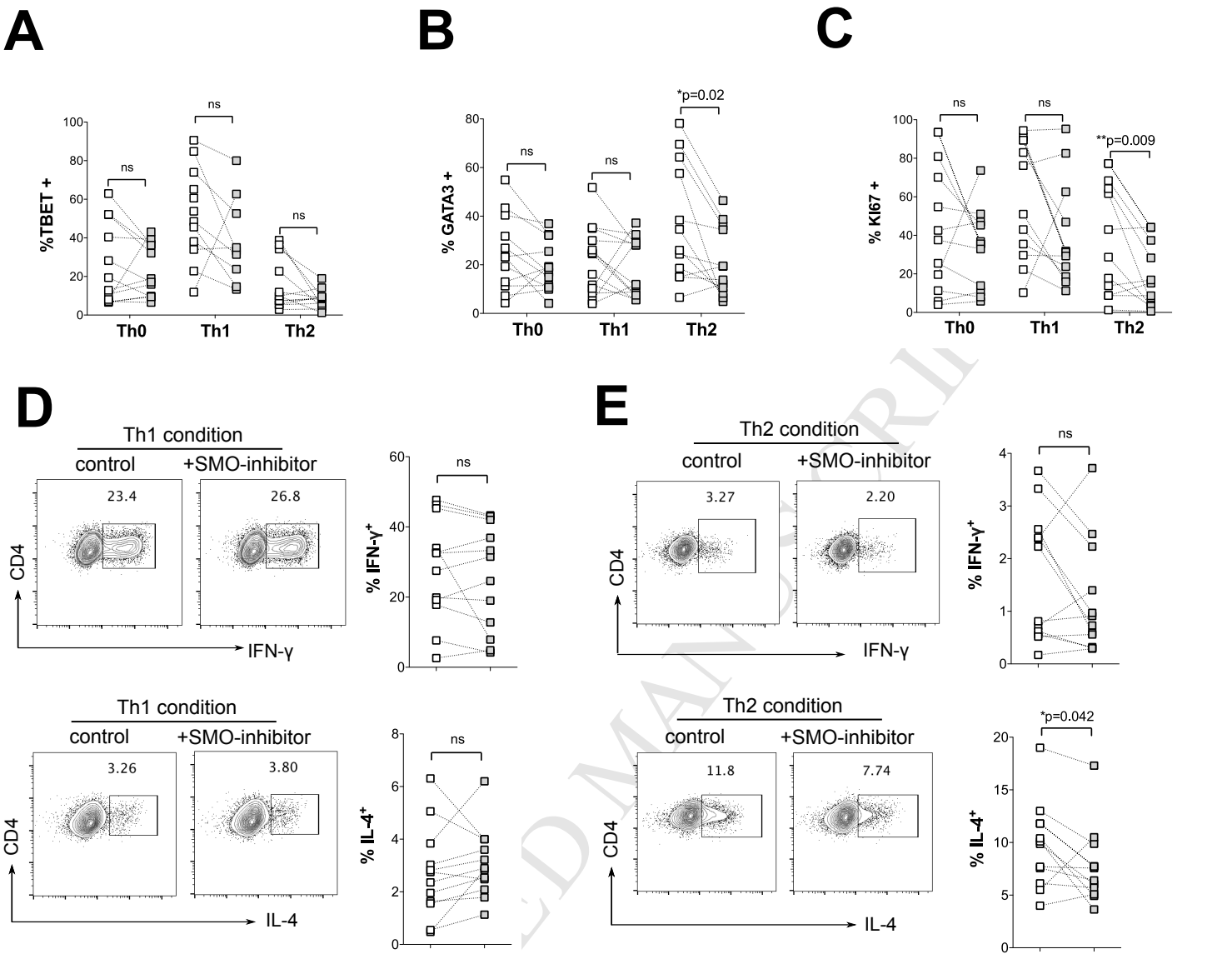


Figure 2



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; Olso, 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 approved 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, naive CD4 T-cells were rested for 3-5 hours and then plated in 96 round well plates at $1x10^6$ cells/ml. Cells were stimulated in complete RPMI (supplemented with 10%FBS, 1% penicilin-streptomycin and 10^{-5}M 2-mercaptoethanol) with 5μg/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 (5μg/ml), rIL-12 (20ng/ml), rIFN-γ (10ng/ml); and for Th2: anti-IFNγ (2.5μg/ml), rIL-4 (20ng/ml) were added. After 4 days, cells were expanded in human rIL-2 (100U/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 (1μg/ml) prior to gene expression and cytokine analysis. Where stated, recombinant Shh (rShh) (R&D Systems) was added at a final concentration of 0.5μg/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; (1µl/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 3µg/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 $p < 0.001$(***).