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Tissue-Derived Hedgehog Proteins Modulate Th Differentiation and Disease

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Genome-wide association studies of complex immune-mediated diseases have indicated that many genetic factors, each with individual low risk, contribute to overall disease. It is therefore timely and important to characterize how immune responses may be subtly modified by tissue context. In this article, we explore the role of tissue-derived molecules in influencing the function of T cells, which, owing to their migratory nature, come in contact with many different microenvironments through their lifespan. Hedgehog (Hh) proteins act as secreted morphogens, providing concentration-dependent positional and temporal cell-fate specification in solid tissues. Hh signaling is required for embryogenesis and is important in postnatal tissue renewal and in malignancy. However, the function of Hh in dynamic, fluid systems, such as in mammalian immunity, is largely unknown. In this article, we show that Hh-dependent transcription in T cells promoted Th2 transcriptional programs and differentiation, exacerbating allergic disease. Of interest, expression of Sonic Hh increased in lung epithelial cells following the induction of allergic disease, and lung T cells upregulated Hh target gene expression, indicating that T cells respond to locally secreted Hh ligands in vivo. We show that Il4, the key Th2 cytokine, is a novel transcriptional target of Hh signals in T cells, providing one mechanism for the role of Hh in Th differentiation. We propose that Hh, secreted from inflamed, remodeling, or malignant tissue, can modulate local T cell function. Our data present an unexpected and novel role for tissue-derived morphogens in the regulation of fluid immune responses, with implications for allergy and tumor responses, suggesting new uses for anti-Hh therapeutics.  

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Hedgehog (Hh) family proteins—Sonic Hh (Shh), Indian Hh, and Desert Hh (Dhh)—are secreted intercellular signaling molecules, essential for embryogenesis and important in the homeostasis of adult tissues (1). Morphogens such as Hh specify cell fate by establishing a concentration gradient, whereby the position of a target cell relative to the source of Hh determines the signal received. The Hh signaling pathway is initiated by ligand binding to the cell-surface receptor Patched 1 (Ptc1), relieving inhibition of Smoothened (Smo), resulting in signal transduction. At the end of the pathway are the Gli family of transcription factors (Gli1, Gli2, and Gli3) (2). Gli proteins bind DNA at consensus Gli-family binding sites and directly modulate target gene transcription. Gli2 is necessary to initiate the signal and acts mainly as an activator but can be processed to activate or repress transcription by posttranslational modification (3). Strength and duration of the signal received, and so outcome, are determined by the balance of intracellular (Ic) Gli-Repressor and Gli-Activator proteins.

Knockout (KO) and transgenic mouse models show that Hh signaling regulates thymocyte development (4–14). Peripheral T cells express components of the Hh signaling pathway (15), which is involved in regulation of T cell activation in vitro (11, 12, 15, 16). Hh family proteins are widely expressed in postnatal tissues, many of which harbor resident T cells, including skin, lung, gut, bone marrow and spleen (17–21). In this study, we tested the hypothesis that Hh-dependent transcription modulates differentiation and effector function of peripheral CD4+ T cells. Naive CD4+ T cells can differentiate down several lineage pathways, with distinct Th functions (22). Th1 and Th2 cells are distinguished by their hallmark profiles of cytokine secretion, lineage-specific transcription factors, and different cellular functions. Th1 cells express Tbet, produce IFN-γ, and control immune responses against Ic pathogens. Th2 cells express Gata3; secrete IL-4, IL-5, IL-9, IL-13, and IL-25; and are important for protection against extracellular parasites. Th2 cells are also involved in the pathogenesis of allergy and atopic disease (23). IL-4, the primary Th2 cytokine, is necessary for the generation of Th2-driven immune responses (24). Conditional deletion of Gata3 from naive T cells blocked differentiation into functional IL-4–secreting cells (25). Expression of Gata3 and that of Il4 are closely linked: Gata3 can directly activate transcription of the Il4 gene (26), but for Th2 differentiation, naive T cells require TCR and IL-4 signaling for strong induction of Gata3 (27). However,
the complex cell-intrinsic and environmental mechanisms that induce upregulation of these key regulators of differentiation are incompletely understood. A large body of research has identified major regulators of Th differentiation, including cytokines, transcription factors, and other immune cell–derived molecules. However, little is known about the contribution of nonimmune factors, including Hh proteins, which are secreted from the cells’ environment, to T cell differentiation and plasticity. Genome-wide association studies (GWAS) into complex immune–mediated diseases have indicated that many genetic factors, each with individual low risk, contribute to overall disease. For asthma, GWAS have shown that multiple loci contribute to allergic disease and drug responsiveness (28, 29) and that several factors may be involved in the communication between epithelial and immune cells in pathophysiological processes (29). It is therefore timely and important to characterize how immune responses may be subtly modified by environmental cues. In this article, we explore the role of tissue context in influencing the function of T cells, which, because of lymphocyte trafficking, come into contact with many different microenvironments throughout their lifespan.

Materials and Methods

**Mice**

Lck-Gli2ΔN2 (11), Lck-Gli2ΔC2 (12), and Dhh KO (30) mice and littermate/age-matched controls, all on C57BL/6 background, were used under U.K. Home Office regulations. The allergic airways model was as described (31).

**Cell culture**

Splenocytes were magnetically purified using the EasySep mouse CD4+ cell negative selection kit (StemCell Technologies). T cells were cultured at 5 × 10^6/ml in AIM V (Invitrogen). For microarray, cells were activated for 6 h with 0.01 μg/ml soluble anti-CD3 and anti-CD28 (BD Biosciences). For chromatin immunoprecipitation (ChIP)/quantitative PCR (qPCR), and anti–IL-4 experiments, CD4+ cells were cultured for 48 h with anti-CD3/ anti-CD28–coated beads (1:1 ratio; Invitrogen). A total of 500 ng/ml recombinant mouse (rm) Shh (R&D Systems) or 5 μg/ml mouse CD4+ cells were cultured with 5 E1 (anti-Hh mAb; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) was used. For 1c cytokine staining, splenocytes were cultured for 3–4 h with 50 ng/ml IL-12 (Gli2R2), 500 ng/ml mononoycin (Sigma-Aldrich), and 3 μg/ml brefeldin A (eBioscience). For Th skewing cultures, 5 × 10^7/ml CD4+ cells were cultured on 5 μg/ml plate-bound anti-CD3 with 1 μg/ml soluble anti-CD28 in complete RPMI 1640 + FCS (Invitrogen) for 6 d. For Th conditions, 10 ng/ml rmLIF, 5 μg/ml anti-IL-4 were added. For Th2 conditions, 10 ng/ml rmIL-4, 5 μg/ml anti-mouse IFN-γ, and 5 μg/ml anti-mouse IL-12 were added (antibodies/ proteins; eBioscience).

**Flow cytometry**

Flow cytometry and reagents were as described (14).

**ELISA**

Th1/Th2 panel or Ready-Set-Go! ELISA kits (eBioscience) were used. Shh ELISA was performed using an Shh DuoSet kit (R&D Systems).

**Histological examination**

Lung tissue was fixed in formalin and embedded in paraffin wax for sectioning. Periodic acid–Schiff (PAS) stains were analyzed by double-blinded scoring. Shh immunohistochemistry was as described (32).

**Microarray and data analysis**

University College London Genomics processed total RNA and acquired data from Affymetrix MOE430 2.0 mouse whole-genome arrays (dataset: GSE33156, Gene Expression Omnibus [GEO] repository). Data were normalized using maas of affy in each dataset. Differentially expressed genes were identified by p < 0.05, considering a false discovery rate by limma (Bioconductor). Principal component analysis (PCA) was performed using the CRAN package. A novel application of canonical correspondence analysis was used to compare our dataset relative to external data (33), whereby a Th1→Th2 axis was generated from GSE14308 (GEO dataset, Affymetrix, Th1/Th2-skewed mouse T cells) with PCA, and used as a gradient for canonical correspondence analysis of our own datasets.

**qRT-PCR**

The qRT-PCR was carried out as described (12, 14). Samples were analyzed in triplicate (13) following normalization to Hprt expression and independently verified in two or three separate experiments.

**Chromatin immunoprecipitation**

SAbioscences ChampionChIP Kit was used. Precleared, sonicated chromatin was immunoprecipitated with anti-Gli2 (Santa Cruz), anti-RNA polymerase II (SABiosciences), or mlg6 (SABiosciences). DNA was purified and used in PCR/qPCR, using primers specific for the HS2 region of Il4 (as primer pair 21 in Ref. 34) and ChampionChIP Gata3 primer assays (SABiosciences). Results were validated in replicate experiments.

**Data analysis**

Statistical analyses were performed using Microsoft Excel or Prism 4 (GraphPad), as stated in the text or figure legends. Significance was reached at p < 0.05.

**Results**

Gli2 modulates Th cell gene expression

Morphogens establish concentration gradients in solid tissues, but the immune system is fluid, with lymphocytes trafficking between blood, lymphoid organs, and tissues. It is not known how morphogens such as Hh influence immune-cell biology. Therefore, to study the effect of Hh signaling on T cells, independent of their history or migratory patterns, we established transgenic models in which transcription by Gli2 is either constitutively activated or repressed in T-lineage cells. Lck-Gli2ΔN2 (Gli2A) mice carry a truncated form of Gli2 that acts as a permanent transcriptional activator of Hh target genes (11). Conversely, Lck-Gli2ΔC2 (Gli2R) mice express a repressor of Gli2-dependent transcription, which, by binding to Gli binding sites, inhibits endogenous Hh-dependent transcription, and hence Hh signaling, in the cell (12). Thus, comparison of transcriptional profiles in wild-type (WT) and Gli2R mice identifies genes whose expression is regulated by physiological Hh signal transduction. Both transgenes are driven by the proximal lck promoter and, as such, are expressed in peripheral T cells (35, 36).

To define the transcriptional response of CD4+ T cells to Hh pathway activation, we examined Hh-dependent gene expression in resting CD4+ cells (unstimulated) and CD4+ cells treated with anti-CD3/CD28 for 6 h (stimulated) from WT, Gli2A, and Gli2R spleen. Hundreds of differentially expressed genes were identified between WT and transgenic groups (GEO ref.: GSE33156). Samples clustered according to genotype (Fig. 1A: unstimulated; Fig. 1B: stimulated), and Hh signaling/responsive genes, Ptx1l and Smo, were upregulated in Gli2A, but not Gli2R (Fig. 1A, cluster II; Fig. 1B, clusters III and IV; Fig. 1B–E), as expected. We validated expression levels by qPCR, using RNA from microarray experiments and independent cell-sorts (Fig. 1D). In agreement with microarray data, relative expression of Ptx1l was 9-fold higher in Gli2A cells than in WT, but was downregulated 2-fold in Gli2R cells compared with WT, indicating that active Hh signaling is taking place in T cells ex vivo.

To identify common effects of Hh signaling in resting and activated T cells, we applied PCA (Fig. 1F). Principal component analysis (PCA) showed that the largest difference in transcriptionomes was between the unstimulated and stimulated samples, indicating that activation stimuli caused profound changes in transcription. PC2, the second largest measure of difference in gene expression, reflected differences between Gli2A and the other samples, especially Gli2R (Fig. 1F), and therefore reflected differences in Hh signaling.
Of interest, when we analyzed gene lists generated by t testing and by PCA, we found that genes involved in Th cell differentiation were differentially expressed between groups. The Th2 cytokine, Il4, was upregulated in Gli2A unstimulated and stimulated cells (Fig. 2A) and belonged to the same clusters as Ptch1, a known Hh target gene (Fig. 1A, 1B, clusters II and III), suggesting that Il4 is downstream of Hh signaling in T cells. In addition, Th1-related genes, including Ifng and Cxcr3, were differentially regulated in Gli2A and Gli2R cells (Fig. 1A, cluster I; Fig. 2A). Stimulated Gli2R cells showed decreased expression of Il4 and Il1rl1, the IL-33R, both important for Th2 function (Fig. 2A). Ptch1, Smo, and Il4 had high PC2 scores, indicating strong association with Gli2A, and therefore active Hh signaling. However, Th1-related genes showed negative PC2 scores, indicating that expression of these genes was increased in Gli2R and suppressed in Gli2A, confirming the results in Fig. 1A.

**Gli2A T cells are Th2-like**

These analyses suggested that Gli2A cells display transcriptional features similar to those of Th2 cells. To test this idea, we used a method with minimal assumptions to generate a scale of Th1/Th2 skewedness (Th1 → Th2 score) based on publically available whole-genome array data derived from Th-skewed cells (GEO database ref.: GSE14308). We found that Gli2A samples showed...
TISSUE-DERIVED Hh MODULATES Th DIFFERENTIATION

We investigated the abundance of Hh pathway proteins in differentiating T cells mediated transcriptional changes that promote Th2 differentiation. We therefore tested this hypothesis experimentally. Together, these analyses suggested that active Hh signaling in T cells following treatment with recombinant rShh, confirming that this effect is Hh specific (Fig. 2E), and was repressed by the addition of neutralizing anti-Hh mAb (5E1) to the cultures (Fig. 2F). Thus, physiological Hh signaling increases IL-4 transcription in WT T cells.

To confirm that Gli2A T cells produce more IL-4 protein, we stimulated lymphocytes in vitro with anti-CD3/CD28 for 72 h and assayed IL-4 secretion by ELISA. Gli2A cells produced, on average, twice as much IL-4 as did WT cells (Fig. 2G).

The Th2 lineage-specific transcription factor Gata3 was not upregulated in Gli2A cells at 6 h (GEO database ref.: GSE33156). However, as Gli2A CD4+ T cells readily upregulated Il4 mRNA expression and cytokine production, we tested whether Gata3 expression was increased in these cells after 48 h of activation. qPCR analysis showed that the presence of Gli2A led to a greater induction of Gata3 than in WT stimulated cells (Fig. 2H).

Gli2A enhances Th2-associated pathological states pathology

Our data suggest that activation of Hh signaling predisposes T cells to become Th2-like upon stimulation. To test whether Hh-dependent transcription enhances Th2 differentiation and function in vivo, we used a murine model of mild allergic asthma, in which dosing with house dust mite (HDM) extract elicits allergic disease (31). After repeated intranasal administration of allergen/PBS, bronchoalveolar lavage fluid (BAL), lung lobes and mediastinal lymph nodes were collected from WT and Gli2A animals. For WT and Gli2A groups, the percentage and number of infiltrating lung CD4+ cells increased upon HDM treatment (Fig. 3A), indicating T cell recruitment during the response. The percentage and number of CD4+ cells in lung were reduced in Gli2A mice compared with WT (Fig. 3A), reflecting the decreased proportions of peripheral T cells in these mice (11). As expected, eosinophils were observed in BAL of HDM-treated mice only. More eosinophils were present in the airways in HDM-treated Gli2A mice compared with WT (Fig. 3B), indicating increased severity of the inflammatory phase of disease. PAS staining showed that the prevalence of mucus-secreting cells in the bronchioles of Gli2A lungs was increased compared with lungs in WT (Fig. 3C, 3D). Of interest, positive PAS staining was seen in PBS-treated Gli2A mice, possibly indicating some underlying airway disease. In addition, BAL samples were analyzed by ELISA for Th2 cytokines IL-4 and IL-13. Increased levels of both Th2 cytokines were found in the HDM-treated Gli2A group relative to WT (Fig. 3E, 3F), despite the decreased percentage and number of CD4+ cells in lung tissue of transgenic animals (Fig. 3A).

To characterize T cells in the allergic airways model, we examined IL-4, IL-13, and IFN-γ production on a per-cell basis by Ic cytokine staining in CD4+ cells. Allergen treatment induced production of Th2 cytokines IL-4 and IL-13 in WT and Gli2A mice, as expected (Fig. 3G, 3H). Following HDM treatment, the proportions of CD4+ cells in lung expressing IL-4 (Fig. 3G) and IL-13 (Fig. 3H) were higher in Gli2A than in WT. IFN-γ was induced by HDM treatment in the WT T cells, but not in the Gli2A group (Fig. 3I). Together these data suggest a Th2-biased response in Gli2A animals. As expected (see Fig. 2), T cells from Gli2A PBS-treated animals showed increased proportions of cells expressing Th2 cytokines, compared with cells from WT (Fig. 3G, 3H). Interestingly, goblet cell hyperplasia (Fig. 3C, 3D) was also observed in this group, suggesting that Hh-mediated transcription favors the Th2 phenotype even without allergen treatment. However, PBS-treated Gli2A mice did not recruit eosinophils into the airway (Fig. 3B), indicating that any basal Th2 inflammation is not equivalent to HDM-induced allergic response. This finding

FIGURE 2. Hh-dependent transcription favors a Th2 transcriptional profile. (A) Gene expression in CD4+ T cells from WT, Gli2A, and Gli2R spleen (n = 3 per group) analyzed by Affymetrix microarray. Expression of Il4, Ifng, Cxcr3, and Il1rl1 in each sample. (B) Samples were measured by Th1→Th2 score; scale generated from publicly available Affymetrix datasets from in vitro–skewed Th1 and Th2 cells. On the scale 0 is the mean value of WT samples. Scale generated from publicly available microarray datasets from Th1 (negative) or Th2 (positive) samples. (C) Representative example qPCR measuring Il4 expression relative to Hprt in CD4+ splenocytes cultured with anti-CD3/CD28–coated beads. (D) The % CD4+IL-4+ splenocytes directly ex vivo by Ic cytokine staining; independent sets of experiments indicated by different-shaped points (n = 5 experiments, unpaired t test: p < 0.02). qPCR for Il4 as in (C); cells cultured with (E) rShh or with (F) anti-Shh (5E1). Mean ± SD Il4 expression per independent sample was calculated relative to WT [unpaired t tests; (E) n = 3, p = 0.04; (F) n = 7, p = 0.03; (F) n = 4, p = 0.02]. (G) Mean ± SD production of IL-4 by ELISA after 72 h in culture with 5 μg plate-bound anti-CD3/CD28 and 20 U/ml IL-2 (WT n = 2; Gli2A n = 3; unpaired t test, p = 0.02). (H) qPCR as in (C) to measure Gata3 relative expression. AU, Arbitrary units.

high, Th2-like scores when cells were either resting or stimulated (Fig. 2B). Interestingly, although the skewed reference data were obtained from T cells that had been cultured for several days in skewing conditions, our transgenic T cells, compared with WT cells, showed a clear bias to Th2 (Gli2A) when prepared fresh from the mouse. After only 6 h of stimulation, Gli2A cells had further increased their Th2 score. Unstimulated Gli2R had a low score on the scale, suggesting a Th1 bias, supported by higher levels of Ifng and Cxcr3, and lower expression of Il1rl1 (Fig. 1A, cluster I; Fig. 2A). However, this trend in global gene expression disappeared upon stimulation (Fig. 2B).

Together, these analyses suggested that active Hh signaling in T cells mediated transcriptional changes that promote Th2 differentiation. We therefore tested this hypothesis experimentally. We investigated the abundance of Hh transcript by qPCR in activated CD4+ T cells from WT and Gli2A spleen and found that Il4 expression in Gli2A cells was ∼6-fold higher than in WT (Fig. 2C). A consistent increase was noted in the proportion of Gli2A cells producing IL-4 directly ex vivo compared with WT, as assessed by Ic cytokine staining on freshly isolated splenocytes

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may indicate an uncoupling of goblet cell formation and IL-4/IL-13 production from eosinophil recruitment.

These data show that Gli1-dependent transcription in T cells exacerbates Th2-mediated allergen-induced disease in vivo. Therefore, Hh proteins released from tissue may signal to local T cells, resulting in enhanced Th2 differentiation or function. This finding would be of particular importance if Hh were released by inflamed tissue, as it would drive a positive feedback loop. To confirm that Hh family member proteins are present in non-transgenic, WT lung and investigate whether Hh expression is upregulated in allergic tissue, we assayed whole-lung tissue homogenate from WT mice following PBS or HDM treatment. Dhh and Shh mRNA was detectable in lung samples by nonquantitative RT-PCR (Fig. 4A). We found that expression of Shh transcript (Fig. 4B, qPCR) and protein (Fig. 4C, ELISA) was significantly upregulated in lung homogenates from HDM-treated mice compared with PBS controls. Immunohistochemistry showed that Shh protein is present at low levels in control lung (Fig. 4D) but is strongly expressed in HDM-treated WT lung, localized to basal stroma of airways, bronchial and alveolar epithelium, and perivascular areas (Fig. 4E). To investigate further, we induced disease in WT mice and sorted epithelial (CD45-EpCAM+) and CD4+ T cells (CD45+CD3+CD4+) from fresh lung by FACS and assessed gene expression by qPCR. In support of immunohistochemistry data, expression of Shh was increased in epithelial cells from HDM-treated compared with PBS-treated WT mice (Fig. 4F). To determine whether lung T cells were actively receiving Hh signals in vivo, we measured expression of Smo, a reliable Hh target gene in T cells (Fig. 1E), in sorted lung CD4+ cells. Smo levels were raised in CD4+ cells from HDM-treated mice versus controls (Fig. 4G), indicating that lung T cells transduced Hh signals in vivo, and that the pathway increased in activity upon induction of allergic disease.

**Hh signals promote Th2 differentiation**

Expression of Gata3 is induced and maintained by Th2-skewing conditions, drives production of Th2 cytokines, occurs over a course of hours to days (37), and can thus be used as an indicator of Th2 transcriptional identity. Therefore, to investigate the molecular mechanisms behind the exacerbation of allergic airways disease caused by Hh-mediated transcription in T cells, we cultured WT and Gli2A CD4+ cells in Th-skewing conditions following activation, and measured commitment to Th2, by lc expression of Gata3 protein.

In neutral Th0 conditions, levels of Gata3 were always higher in Gli2A than in WT CD4+ cells (Fig. 5A, 5B), suggesting an inherent bias toward Th2. In Th2 conditions, in which exogenous IL-4 is added, WT cells displayed Gata3 levels similar to those in Gli2A cells at 24 h and 48 h, suggesting that enhanced expression of Gata3 by Th0-conditioned Gli2A cells is the result of their increased IL-4 production. However, after 6 d in culture, on average, double the proportion of Gli2A cells expressed Gata3, compared with WT cells, showing enhanced commitment to the Th2 lineage (Fig. 5A, 5C).

Th1 differentiation is controlled by Tbet, which antagonizes the effects of Gata3 and vice versa (38). To test whether Gli2A cells displayed decreased Th1 potential compared with WT, we examined Tbet expression by staining in skewing cultures at 48 h. Expression of Tbet was high and similar in Th0- or Th1-conditioned cultures, but in Th2 conditions was decreased in Gli2A cells compared with WT cells (Fig. 5D). This finding resulted in an increased ratio of Gata3/Tbet, particularly in Th2-skewed Gli2A CD4+ cultures (Fig. 5E), confirming that these cells are biased toward a Gata3+ Th2 phenotype.

To verify that Hh signaling promotes Th2 differentiation of nontransgenic cells, we cultured WT CD4+ cells in neutral Th0 conditions for 72 h with a single dose of rShh. By 48 h, Gata3 expression was higher in Hh-treated cells (Fig. 5F, 5G). To ask if the increase in Gata3 expression on rShh treatment was the result of increased IL-4 production, we cultured WT CD4+ cells in Th2 conditions in the presence of rShh. As expected, when IL-4 was added to the cultures, Gata3 expression was not affected by rShh (Fig. 5H), suggesting that strong IL-4 signals in the cultures (also in the presence of neutralizing anti-IFNγ) are sufficient for maximal Th2 differentiation, so Shh treatment had no impact, or that IL-4 induction is downstream of Shh signaling.

**Repressing physiological Hh inhibits Th2**

To test whether repression of physiological Hh-dependent transcription would impair Th2 potential and suppress Gata3 expression, we performed in vitro skewing experiments using Gli2R CD4+ cells. Gata3 did not reach WT levels over the time course in...
Th0 (Fig. 6A, 6B) or Th2 (Fig. 6A, 6C) conditions, indicating that repression of physiological Hh pathway activation in T cells reduces their ability to maximally upregulate Gata3 and differentiate to the Th2 lineage. The fact that addition of exogenous IL-4 and anti–IFN-γ (Th2 conditions) to Gli2R cultures was not sufficient to restore Gata3 expression to WT levels indicates that Hh-dependent transcription upregulates expression of Gata3 and skews cells toward a Th2 phenotype in vitro. (A) Ic Gata3 protein expression in WT and Gli2A CD4+ splenocytes after culture in Th-neutral (Th0) or Th2-skewing conditions. Mean ± SEM (B) %Gata3+ cells as fold change (FC) of the WT cultures (n = 3 independent experiments) in (B) Th0 (unpaired t test, *p = 0.01, **p = 0.001) and (C) Th2 conditions (unpaired t test, *p = 0.02). (D) Ic Tbet protein in Th0 or Th1/Th2-skewed cells. (E) Example Gata3/Tbet ratio at 48 h. (F) WT CD4+ cells cultured for 3 d with a single dose of rShh at the start of the culture in (G) Th0 neutral (n = 5, paired t test: %Gata3+, p = 0.04; mean fluorescence intensity, Gata3, p = 0.03) and (H) Th2 conditions (n = 5). Dotted overlays show example Ic isotype control staining; mean fluorescence intensity of each histogram is shown in italics.

![Image](https://via.placeholder.com/150)

**FIGURE 5.** Active Hh-dependent transcription upregulates expression of Gata3 and skews cells toward a Th2 phenotype in vitro. (A) Expression of Dhh, Indian Hh (Ihh), and Shh by nonquantitative RT-PCR in homogenized lung tissue from PBS- or HDM-treated WT BALB/c mice. Blk, water blank; EH, embryo head positive control. (B) Representative triplicate qPCR showing mean ± SD expression of Shh transcript in lung relative to Hprt [mean ± SEM relative Shh expression in PBS- (n = 4) compared with HDM-treated (n = 6) WT mice: p = 0.02]. (C) Mean ± SEM Shh protein concentration (ELISA, p = 0.04) in homogenized lung of WT mice treated with PBS (n = 4) or HDM (n = 6). Immunohistochemical detection of Shh in (D) PBS- and (E) HDM-treated WT fixed paraffin-embedded lung sections at ×100 (upper panels) and ×400 (lower panels) magnification. Arrowheads indicate Shh staining. (F and G) Lung epithelial cells (CD45-EpCAM+) and lung CD4+ T cells (CD45−CD3+CD4+) were sorted by FACS from WT mice treated with PBS or HDM. Representative triplicate qPCR showing mean ± SD expression of (F) Shh and transcript in lung epithelial cells and (G) Smo in lung CD4+ T cells, relative to Hprt. Mean ± SEM relative Shh expression in PBS- (n = 3) compared with HDM-treated (n = 3) independent lung epithelial cell samples from WT mice: p = 0.0095; for Smo in lung CD4+ cell samples: p = 0.02.

To confirm that the activation or inhibition of Hh-dependent transcription altered IL-4 production by cells cultured in skewing conditions, we measured IL-4 protein concentration by ELISA after 6 d in culture (Fig. 6D). As expected, cultures of Gli2A cells contained, on average, 2.8-fold more IL-4 than did WT cells, and Gli2R cells half the amount of IL-4 after culture in Th0 conditions (Fig. 6D, upper panel). In Th2 conditions, compared with WT, Gli2A and Gli2R culture supernatants contained 1.5-fold and 0.5-fold IL-4, respectively (Fig. 6D, lower panel). Similar levels of IL-4 were detected in supernatants from Th0 conditions as in supernatants from equivalent Th2-skewed cultures. This observation has been made before (39) and most likely reflects both the fact that Gli2A cells secrete high levels of endogenous IL-4 and the complex balance between the kinetics of IL-4 production, uptake, and degradation during the prolonged 6-d culture period in different conditions.

Physiological levels of Hh pathway activation in T cells isolated fresh from the mouse spleen thus skew transcriptional processes to favor Th2 differentiation (Fig. 6). Given this, we examined the effect of reducing environmental Hh protein in the spleen on Th2 differentiation of nontransgenic CD4+ cells ex vivo. We used Dhh KO CD4 splenocytes, as Dhh is expressed by spleen stroma (40) and, unlike Shh KO, is not an embryonic-lethal mutation. We cultured Dhh KO and WT cells in Th2 skewing conditions and found that Dhh KO cells upregulate Gata3 less efficiently than do WT cells after stimulation (Fig. 6E). This finding was statistically significant.
FIGURE 6. Physiological Hh signaling controls expression of Gata3 and Il4. (A) Ic Gata3 staining as in Fig. 5 in WT and Gli2R CD4+ splenocytes in (B) Th0 (mean ± SEM, n = 3, *p = 0.004, **p = 0.009) and (C) Th2 conditions (‡p < 0.05, *p = 0.005). (D) IL-4 production per 10^5 cells measured by ELISA after 6 d in culture in Th0 (upper panel) or Th2 (lower panel) conditions (WT, n = 3; Gli2A, n = 2; Gli2R, n = 2; in triplicate, ‡p < 0.01). (E and F) Ic Gata3 in Dhh KO and WT CD4+ cells (n = 3, *p = 0.0004) in Th2 conditions. Dotted overlays show control stain; mean fluorescence intensity of each histogram is shown in italics. (G) Mean ± SD Gata3 expression relative to Hprt in activated CD4+ splenocytes from Gli2R, or WT ± anti-Hh (5E1); triplicate qPCR representative of two independent experiments (WT versus Gli2R, WT versus WT ± anti-Hh: p < 0.05). (H) The murine Il4 locus. (I and J) Sonicated chromatin from activated Gli2A CD4+ splenocytes was immunoprecipitated with anti-Gli2, control IgG, and anti-RNA-PolII, and ChIP-PCR was performed on the intronic HS2 region of Il4. (I) End-point PCR showed a product in input (nonimmunoprecipitated) and Gli2-precipitated fractions. (J) Fold enrichment in the Gli2-precipitated fraction was calculated based on input and control, quantified by qPCR. at 24 h (Fig. 6F), suggesting that fresh KO cells are inherently impaired in their ability to undergo rapid Th2 differentiation, as a result of reduction in Hh signal from their environment.

Given that Gata3 protein expression was lowered by a reduction in Hh signaling, we measured Gata3 mRNA in Gli2R CD4 cells and in WT cells treated with anti-Hh mAb after 48 h of anti-CD3/CD28 stimulation. Gata3 transcription was reduced by inhibition of Hh signaling (Fig. 6G). Taken together, these experiments show that the physiological Hh signal can functionally control expression of this lineage-specifying transcription factor in CD4+ splenocytes.

Gli2 binds the Il4 gene

The rapid (6 h) induction of Il4 (Fig. 1), and increased IL-4 production (Figs. 2, 6) by Gli2A cells, altered Gata3 induction (Figs. 5, 6), and marked repression of Gata3 expression in Gli2R CD4+ T cells and 5E1-treated WT cells (Fig. 6G) prompted us to investigate whether Gli2 could act directly to initiate or enhance transcription of Il4 and/or Gata3. We examined genomic sequences for suggested Gli consensus binding sequences and found several potential sites in Gata3 and Il4 genomic sequences. We therefore investigated whether Gli2 can directly bind Il4 and Gata3 by ChIP. CD4+ cells from Gli2A mice were stimulated for 48 h, and then fragmented chromatin was immunoprecipitated with anti-Gli2 or control Abs. DNA was purified from bound targets, and PCR was performed to amplify regions of the Gata3 and Il4 genes identified as containing potential Gli binding sites. We found no enrichment of Gata3 by conventional PCR or qPCR assays, which span 1 kb up- and downstream of the transcription start site, incorporating regions containing possible Gli binding sites. However, in the case of the Il4 locus (Fig. 6H), we observed significant binding to a region localizing to an important enhancer element in intron 2 (DNAse-hypersensitivity site HS2; Ref. 41) (Fig. 6I, 6J), but not to regions around the Il4 promoter (not shown). The HS2 region is critical for full IL-4-dependent Th2 responses (34) and is necessary for chromatin remodeling essential for lineage-specific Il4 transcription of this lineage-specifying transcription factor in CD4+ splenocytes.

FIGURE 7. Hh regulates Th2 differentiation. (A) Ic Gata3 staining in CD4+ splenocytes after 48 h in culture with anti-CD3/CD28-coated beads either with (+) or without (−) 5 μg/ml anti-IL-4 mAb. Histograms represent at least two independent experiments; mean fluorescence intensity is shown in italics, and percentages refer to proportion of cells to the right of the marker. (B) Ic cytokines measured after 6 d in culture ± anti-IL-4, as described in (A). Markers on dot plots denote %cells with positive staining, as assessed by isotype control (not shown); italics indicate mean fluorescence intensity of population. (C) Schematic of a proposed model in which inflamed/injured/activated tissue releases Hh protein, signaling to local T cells, causing upregulation of Il4 and influencing other Th genes by Gli-dependent transcription. IL-4 is released, leading to enhanced Th2 differentiation or survival via Gata3 upregulation, and an increase in Th2 function, exacerbating disease and tissue damage.
Tissue-Derived Hh Modulates Th Differentiation

Expression. Our data therefore identify Il4 as a novel target gene of Gli2-dependent Hh signaling and provide a mechanism for the role of Gli2 in skewing Th differentiation.

To test whether the Hh-dependent induction of Gata3 requires Il4, we cultured CD4+ cells with anti-CD3/28-coated activator beads, with or without anti-IL-4 Ab, and measured Gata3 expression after 48 h. As expected, inhibition of IL-4 signaling in WT cultures markedly decreased Gata3 expression, confirming that IL-4 is required to support Th2 differentiation. Activation of Hh signaling enhanced Gata3 expression in CD4+ cells, but this effect was lost when anti–IL-4 was present in the culture (Fig. 7A), confirming that IL-4 is required to induce Gata3 in WT cells and demonstrating that the Gli2A transgene does not activate Gata3 transcription independently of IL-4.

To test the impact of neutralization of IL-4 on the cytokine production of Gli2A cells, we measured IL-4 expression by Ic-cytokine staining in cells after 6 d of stimulation with activator beads. The presence of anti–IL-4 in the culture significantly blocked IL-4 expression (Fig. 7B), indicating that autocrine signaling is required for full IL-4 production. In contrast, the expression of IFN-γ was unaffected by the presence of anti–IL-4.

Discussion

In this article, we demonstrate that expression of Hh ligand increases in tissue following induction of Th2-mediated disease, and that Hh signaling in T cells promotes Th2 function by skewing global transcription of T cells toward a Th2-like profile, directly enhancing IL-4 production. In asthma, this process is pathological, but it may have evolved as an anti-inflammatory mechanism, or to enhance immunity to parasitic infection. Our data have important consequences for understanding how nonimmune, tissue-derived factors influence T cell immunity, particularly given the wide tissue expression of Hh family proteins. We demonstrate that morphogens can play a surprising role in the regulation of fluid, as well as solid organ systems. Highly migratory cells such as lymphocytes will traffic through many microenvironments in the course of their lifespan, each of which therefore having the potential to alter the cells’ function. The role of context-dependent, tissue-derived signals in influencing immunity in disease is an exciting emerging field. Understanding the impact of such events will be important for dissecting the mechanisms controlling complex diseases, which may have several subphenotypes, in which multiple signals integrate to drive pathological processes.

In lung, we have shown that Hh proteins are present under normal conditions and that Shh expression is upregulated in lung epithelial cells during allergic inflammation. Further, CD4+ T cells from lung express enhanced levels of SMO transcript after HDMD treatment, indicating active Hh signaling. Thus, inflamed tissue, including but probably not limited to epithelia, releases Shh, potentiating Hh signaling to local T cells. This potentiation of signaling leads to transcriptional changes, increased IL-4 production, and enhanced Th2 responses, signaling to other immune effector cells, maintaining allergic inflammation, and further aggravating disease (Fig. 7C). It will be of interest to measure Hh levels in BAL or lung from asthma sufferers. Importantly, recent GWAS have identified key single-nucleotide polymorphisms in the Hh-sequestering molecule HHIP as important for lung capacity and function (spirometric measures) in human asthma (42). Our data demonstrate that Hh signaling is also involved in lymphocyte function in murine asthma. Therefore, it is timely to investigate the potential therapeutic use of small-molecule inhibitors of Hh signaling in Th2-mediated disease.

Our study also investigated the influence of Hh proteins on normal T cell differentiation. Hh signaling is involved in adult stem cell renewal and in the pathogenesis of several cancers. To understand and develop strategies to treat Hh-dependent hematological and lymphoid malignancies (43, 44), it is clearly important to understand how Hh proteins function in healthy cells. We identified Il4 as a transcriptional target of Hh signaling in T cells, and this is of interest not only because of its pivotal role in Th2 biology, but also because of the function of IL-4 in signaling to other immune cells. Increased IL-4 in tumor microenvironments can inhibit antitumor responses or promote tumor growth (45, 46). Of interest, increased IL-4 has been observed in several cancers strongly associated with aberrant Hh signaling (47–49), and IL-4 derived from tumor-resident T cells sustained growth of follicular B lymphomas (50), tumors known to produce Hh ligands (51).

Future work should therefore characterize the role of Hh in human allergic disease, as well as investigate whether Hh plays a role in modulating other Th responses and tumor immune evasion.

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Disclosures

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