Indian hedgehog (Ihh) both promotes and restricts thymocyte differentiation

Running title: Ihh in thymus homeostasis

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Non-standard Abbreviations: Ihh (Indian Hedgehog), Shh (Sonic Hedgehog), Dhh (Desert Hedgehog), Gli (Glioblastoma-associated protein), DN (Double Negative), DP (Double Positive), SP (Single Positive), ISP (Immature Single Positive), FTOC (Fetal Thymus Organ Culture), E (Embryonic day)

Summary

We show that Ihh regulates T-cell development and homeostasis in both fetal and adult thymus, controlling thymocyte number. Fetal Ihh-/- thymi had reduced differentiation to DP cell and reduced cell numbers compared to WT littermates, and analysis of Ihh-/-Shh+/- embryos revealed a redundant function for Ihh, promoting the transition from DN1 to DN2. Surprisingly, fetal Ihh+/- thymi had increased thymocyte numbers and proportion of DP cells relative to WT, indicating that Ihh also negatively regulates thymocyte development. In vitro treatment of thymus explants with exogenous recombinant Hedgehog protein promoted thymocyte development in Ihh-/- thymi, but inhibited thymocyte development in Ihh+/-, confirming both positive and negative regulatory functions of Ihh. Analysis of Rag-/-Ihh+/- thymi, in which the pre-TCR signal may be simulated by treatment with anti-CD3 antibody, showed that Ihh promotes T-cell development prior to pre-TCR signaling, but negatively regulates T-cell development only after pre-TCR signaling has taken place. We show that *ihh* is most highly expressed by the DP population, and that Ihh produced by DP cells feeds back to negatively regulate the differentiation and proliferation of their DN progenitors. Thus, differentiation from DN to DP cell, and hence the size of the DP population is dependent on the concentration of Ihh in the thymus. Analysis of Ihh conditional knockout and heterozygote adult mice showed that Ihh also influences DP cell production and thymocyte number in the adult.

Introduction

Here we show that the intercellular signaling molecule, Indian Hedgehog (Ihh) regulates T-cell development, thereby restricting thymocyte number.

Thymus size is tightly controlled by processes intrinsic to the thymus, about which little is known. The control of thymocyte number has been assumed to rely on competition between thymocyte precursors for limiting concentrations of mitogenic or survival factors, that by positively regulating the fate of the progenitor cell population control the number of thymocytes produced. Many factors have been described that promote the expansion of thymocyte progenitors and promote T-cell development, including cytokines[1], Notch signaling[2,3], the Wnt protein family[4], and Sonic Hh (Shh)[5]. Little, however, is understood about mechanisms that provide feedback, or a counting system, negatively regulating the upper limits of thymocyte differentiation and number. Here we show that Ihh provides such a counting system, negatively regulating the rate of differentiation from CD4-CD8- Double Negative (DN) to CD4+CD8+ Double Positive (DP) cell, and hence T-cell production and thymus size.

The Hh protein family (Shh, Ihh and Desert Hh (Dhh)) signals for development, patterning and organogenesis of many tissues during mammalian embryogenesis[6,7], and is also involved in homeostasis and renewal of adult tissues, including blood and thymus[8-11]. They can act as classical morphogens, giving concentration-dependent signals for position and patterning, and can regulate cell survival and proliferation[6,7]. The three Hh proteins have distinct temporal and tissue-specific expression patterns and functions[12,13]. While Shh and Ihh are each essential during embryogenesis and have some overlapping functions[14], Shh is more pleiotropic and non-redundant in its actions, whereas Ihh has

specialized functions in bone, cartilage and gut[13-15]. The Hh proteins share a common signaling pathway. They bind to the receptor Patched (Ptc), which releases the signal transduction protein Smoothened (Smo) to transmit a signal into the cell. In the absence of Hh, Ptc inhibits the activity of Smo. The downstream components of the signaling pathway are the Glioblastoma-associated protein (Gli) family of transcription factors: Gli1, Gli2 and Gli3[7,16].

During αβ T-cell development CD4-CD8- double negative (DN) cells give rise to the CD4+CD8+ double positive (DP) population, which differentiate to mature CD8+CD4- or CD8$^-$CD4+ single positive (SP) cells. The DN population can be further subdivided by cell surface expression of CD25 and CD44. CD44+CD25- (DN1) cells differentiate to become CD44+CD25+ (DN2) cells, which then differentiate to become CD44-CD25+ (DN3). The DN3 population gives rise to the CD44-CD25- (DN4) subset, which undergo a phase of rapid proliferation before differentiation into the DP population, in general via a cycling immature CD8+ intermediate single positive (ISP) cell. T-cell lineage specification and TCRβ chain rearrangements occur in the CD25+ (DN2 and DN3) population. Pre-TCR signaling is necessary for differentiation to DP cell[17], but other largely unidentified signals dependent on normal thymus architecture and cellular composition are also required[18].

Shh, Ihh and components of the Hh signaling pathway are expressed in the mouse thymus[10,19-23]. *In vitro* studies first demonstrated that Hh signaling influences thymocyte development[10,24], and although they did not determine which Hh protein (Shh or Ihh) was physiologically significant, suggested that Hh signaling was predominantly a negative regulator of T-cell development. In contrast, subsequent *ex vivo* analysis of Shh-/- thymi

revealed multiple positive-regulatory functions for Shh during fetal T-cell development. Absence of Shh caused reductions in thymocyte number; DN cell proliferation; differentiation from DN1 to DN2 cell; survival of DN4 cells; production of DP cells[5]; and the ratio of mature CD8:CD4 SP cells[22,25,26]. Conditional deletion of Smo from T-lineage cells has also shown that the Hh pathway provides essential positive signals for homeostasis of the earliest DN subsets and for differentiation from DN1 to DN2, but did not reveal any influence of Hh signaling on T-cell development after the DN2 stage[21]. Here we take a genetic approach to assess the function of Ihh during thymocyte development, thereby reconciling conflicting data from earlier experimental systems[5,10,21,23,24]. We demonstrate, by analysis of null and conditional-null mutants, that Ihh, produced by thymocytes themselves, regulates T-cell development and homeostasis in fetal and adult thymus. We show that Ihh and Shh have distinct but overlapping functions in the thymus, and that while Shh, secreted by the epithelium, is dominant in positively signaling for proliferation and differentiation of early DN progenitors, Ihh, produced by thymocytes, functions to control thymocyte numbers by negatively regulating the transition from icTCRβ+DN3 to DP, providing concentration-dependent feedback on the production of DP cells.

Materials and Methods

Mice

C57BL/6 mice (B & K Universal Ltd (U.K.), Shh+/-[12], and Ihh+/- [13]mice, gifts from Philip Beachy and Andrew McMahon respectively were backcrossed onto C57BL/6 mice for >11 generations. Cre transgenic mice[27], a gift from Dimitris Kioussis, and Floxed Ihh mice[15], were bred and maintained at Imperial or University College London, under UK Home Office regulations. Timed-mates were as described[5]. All animal experiments were approved by the institutional review board of University College London.

FTOC

FTOC were as described[5]. Where stated, FTOC were treated with Recombinant mouse Sonic Hedgehog N-Terminus protein (R & D Systems, catalog number 464-SH) or 1μg/ml azide-free anti-CD3 (Pharmingen).

Flow cytometry and antibodies

Thymi were dissected, and cell suspensions were prepared, stained and analyzed as described[5,28], using directly conjugated antibodies from BD Pharmingen. Data are representative of >three experiments. Statistical analysis was unpaired Students-t test (equal or unequal variance depending on data) and F-test. To allow comparison between litters, the number of cells recovered from each thymus, or the percentage of cells staining positive with a given antibody, were divided by the mean value from WT thymi from the same litter, to give a relative value or relative cell number. At least three different litters of any embryonic day were analysed.

Genotyping and polymerase chain reactions (PCR) analysis

Ihh-/- embryos die around birth, and from E16.5 can be identified phenotypically by their shortened limbs and slightly small size, whereas Ihh+/- embryos and adults are healthy and phenotypically indistinguishable from WT[13]. In addition to phenotypic identification, all embryos and animals were genotyped by PCR.

DNA extraction and PCR analysis were as described[5], using ~ 0.5μg genomic DNA as template, on a Stratagene Robocycler. Primers:

Ihh/neoForwardAGGAGGCAGGGACATGGATAGGGTG,

ReverseTACCGGTGGATGTGGAATGTGTGCG.

Shh/neoForwardCTGTGCTCGACGTTGTACTG ,

ReverseAAGCCCGAGACTTGTGTGGA.

CreForwardAGATGCCAGGACATCAGGAACCTG,

ReverseTACCGGTGGATGTGGAATGTGTGCG

IhhWT and Ihhfl/fl as described[13].

Real-time RT-PCR

RNA extraction and cDNA synthesis were as described[23]. One primer for each pair was designed to span exon-exon boundaries to avoid amplification of genomic DNA.

Primers: IhhForwardCGACATCATCTTCAAGGACG,

ReverseGTCACCCGCAGTTTCACAC; HPRT and Gli1 as described[22].

Quantitative PCR of VDJ TCRβ rearrangement

TCRβ VDJ rearrangement measurement was carried out as described[29].

Results

Expression of *ihh and gli1* in thymus populations

We have described *ihh* expression in the whole thymus[10]. Here we assess expression of Ihh in FACS sorted E16.5 fetal thymocyte populations and fetal thymus stroma. We found *ihh* expression in both thymus stroma and thymocytes, with highest expression in the DP population. In the sorted thymocyte populations, low *ihh* expression, equivalent to that found in the stroma, was detectable in the DN3 population, and relative expression increased six-fold between the DN3 and DP populations (Fig. 1a).

Both RT-PCR analysis and cell-surface staining have shown that Smo expression is highest in the CD25+DN population, and down-regulated in the subsequent DN4 and DP populations, indicating that the DP cells are unlikely to be able to respond to a Hh signal[10,21], and DP cells are not responsive to Shh treatment *in vitro*[10]. The transcription factor Gli1 is a ubiquitous transcriptional target of Hh signaling, but is not necessary to initiate the Hh signal, and measurement of its transcription is used as a read-out of Hh signaling in a cell population[30]. Therefore, to determine which fetal thymocyte populations responded to the Hh signal *ex vivo*, we assessed *gli1* transcription in sorted fetal thymocyte populations. As predicted by the pattern of Smo expression, we found highest *gli1* transcription in the CD25+ DN populations (DN2 and DN3), and *gli1* transcription was down-regulated in the subsequent DN4 and DP populations (Figure 1b). This pattern of expression correlates well with the expression of *gli3* and *gli2* in thymocytes, which are both expressed in DN populations but down-regulated at the DP stage, confirming that DP thymocytes cannot transduce the Hh signal[21-23]. Thus, the DP population had the highest expression of *ihh*, but did not express significant levels of *gli1*,

implying that they were not themselves responding to an autocrine Hh signal, but rather signaling back to an earlier cell (Fig 1a and b).

Ihh has a redundant function at the transition from DN1 to DN2

To assess the function of Ihh during fetal T-cell development, we studied thymocyte development in Ihh-/- embryos. Given that Smo, Shh and Gli3 are important for differentiation from DN1 to DN2[5,21,23], we investigated a role for Ihh at the same developmental checkpoint. We analysed E13.5 and E14.5 thymi, when the DN1 to DN2 transition first occurs. On E13.5, Shh-/- thymi had a reduction in cell number, proportion of CD45+ cells and differentiation from DN1 to DN2[5]. In contrast, we found no differences in the percentage of CD45+ cells or the proportion of DN1 and DN2 populations between Ihh-/- and littermate thymi (Fig. 1c and d***).*** We likewise found no differences in cell surface expression of CD117 and B220 on the CD45+CD44+ thymocytes between Ihh-/- and littermate thymi (Fig. 1e and f***).*** On E14.5, the Shh-/- thymus contained approximately one tenth the number of thymocytes of its WT littermates, and the size of each thymus lobe was greatly reduced (Fig. 1g, i**).** The E14.5 Ihh-/- thymi, however, were not obviously smaller than WT littermate thymi (Fig. 1g, ii and h). Given that we did not detect an essential function for Ihh at this stage of thymopoiesis, and Shh and Ihh have overlapping functions in other tissues[6,7,14], we assessed redundancy between these two factors. We analysed early thymocyte development and differentiation from DN1 to DN2 in double mutants. Shh-/-Ihh-/- double knock-out embryos die in utero at E9.5[14], so we analyzed E13.5 Shh+/- Ihh-/- and littermate thymi. On E13.5 the Shh+/- thymus is phenotypically normal. Deletion of one copy of *shh* in Ihh-/- (Shh+/- Ihh-/-) did not affect the proportion of CD45+ cells, but did,

however, reduce the proportion of DN2 cells, from 27% in a Ihh-/-Shh+/+ thymus, to 6.9% in the Ihh-/-Shh+/- thymus (Fig. 2a and b). We found no differences in cell surface expression of CD117 and B220 on the CD45+CD44+ DN1 thymocytes between Ihh-/-Shh+/- and littermate thymi (data not shown).

These data reveal a function for Ihh at the DN1 to DN2 transition, and indicate that in the E13.5 Ihh-/- thymus, Shh can compensate for absence of Ihh, but that overall concentration of Hh protein is limiting. As we did not detect *ihh* transcription in the fetal DN1 and DN2 populations (Fig. 1a), the function of Ihh on E13.5 is likely to be mediated by Ihh produced by the thymic stroma. Given that the proportion of CD45+ cells was not reduced by absence of one copy of *shh*, we found no evidence that Ihh is involved in the seeding of the thymus or expansion of early progenitor cells. This function seems to be unique to Shh.

Ihh negatively regulates the transition from DN to DP

To ask if Ihh is important at the transition from DN to DP cell, we compared thymocyte development in E16.5 Ihh-/-, +/- and WT littermates. As E16.5 is the day of embryonic development on which DP cells first appear, the transition from DN to DP on E16.5 is largely synchronized. Thymocyte number was reduced in Ihh-/- thymi to less than half of WT littermate thymi, but surprisingly Ihh+/- thymi contained on average 1.4 times more thymocytes than WT thymi, and there was greater variation between individual Ihh +/- thymi (Fig. 3a). The differences in thymocyte number relative to WT were statistically significant for both Ihh-/- and Ihh+/- embryos and were reflected in the size of the thymus lobes (Fig. 3b).

In Ihh-/- thymi, the proportion of DP cells was reduced (Fig. 3c and d). In Ihh+/- thymi, however, both the proportion and absolute number of DP cells was increased, compared to WT, and on average the Ihh+/- thymus contained 2.4 times more DP cells than WT thymi (Fig. 3c and d). 6.2% of cells were DP in Ihh-/- thymi compared to 10.4% and 17.3% in WT and Ihh+/- littermate thymi respectively (Fig. 3d). Given that the increase in production of DP cells in the Ihh+/- thymus was surprising, we used Quantitative (Q)RT-PCR analysis of RNA from embryonic DP thymocytes to confirm expression levels of *ihh* in WT, Ihh+/- and Ihh-/-. As expected *ihh* transcription was reduced two fold from WT to Ihh+/- and was not detectable in Ihh-/- (Fig. 3e).

Although the reduction in DP cells in the Ihh-/- thymus showed that Ihh promotes T-cell development (as seen in E13.5 Ihh-/-Shh+/- thymi, Fig. 2b), the opposing phenotypes of the -/- and +/- mutants, indicated that Ihh also negatively regulates the transition from DN to DP, as reducing the concentration of Ihh to one half of that of the WT thymus increased the production of DP thymocytes by more than two fold (Fig. 3c-d).

Ihh is not required for TCRβ locus rearrangement

We found an overall increase in percentage of DN cells, but a significant decrease in the proportion of DN4 cells in the Ihh-/- thymus (Fig. 3f). Since transition to the DP stage requires pre-TCR signaling, the reduced differentiation from DN3 to DP observed in Ihh-/- thymus could be the result of failure to rearrange the TCRβ chain locus. To test this, we assessed the ability of DN3 thymocytes to produce functional TCRβ chain protein by measuring intracellular (ic) TCRβ chain expression. The Ihh-/-DN3 population expressed icTCRβ and the percentage of icTCRβ+ cells in the DN3 population was in fact marginally higher in the Ihh-/- embryos compared to WT littermates (Fig. 3g-h). We

confirmed that TCRβ locus rearrangement was efficient in the Ihh-/- thymus, by quantitative PCR[29] using 5' primers to Vβ8.2 and Vβ5.1 and a 3' primer to Jβ2.7 (Fig. 3i). We found no evidence for reduction in efficiency of TCRβ locus rearrangement between Ihh-/- and WT. As successful *TCRβ* locus rearrangement is associated with release from cell cycle arrest[31], we also assessed cell cycle status by PI staining of sorted DN3 cells from WT and Ihh-/- littermates. We found no significant difference in the proportion of cells in S+G2/M (Fig. 3j). We therefore found no evidence for a reduction in TCRβ locus rearrangement.

Ihh is a negative regulator of DN3 cell proliferation

To identify the target cell of Ihh's negative regulation of cell number we sorted CD25+DN, CD8ISP and DP populations from E16.5 Ihh+/- and WT thymi and assessed cell cycle status by PI staining. There was no difference in the percentage of cells in cycle between the DP populations, but the Ihh+/- CD25+DN population contained significantly more cells in S/G2+M than its WT counterpart, identifying it as a target of Ihh's negative regulation of expansion and differentiation (Fig. 4a). In addition, a small but significant increase in the proportion of cells in cycle was observed in the Ihh+/- CD8ISP population, relative to WT (Fig. 4a).

Ihh promotes DN thymocyte development before pre-TCR signal transduction, but is a negative regulator after pre-TCR signal transduction.

As Ihh provides both positive and negative regulatory signals for the differentiation and proliferation of DN thymocytes, we asked when in thymocyte development the positive and negative signals occur in respect to pre-TCR signaling. We compared cell number between Ihh+/+Rag-/- and Ihh+/-Rag-/- thymi and found that the Ihh+/-Rag-/- thymi

were significantly smaller than those of Ihh+/+Rag-/- littermates (Fig. 4b, p=0.0013), demonstrating that Ihh transmits the positive signal before pre-TCR signal transduction. This is consistent with its early function at the transition from DN1 to DN2.

We then tested if Ihh+/-Rag-/- thymocytes expand more efficiently than their Ihh+/+Rag-/- counterparts after FTOC treatment with anti-CD3, thereby mimicking a signal through the pre-TCR. For each thymus, we treated one lobe with anti-CD3 mab for 5 days, and calculated the fold increase in thymocyte number relative to the number of cells in the other untreated lobe from the same thymus. The number of thymocytes in Ihh+/-Rag-/- FTOC increased on average 13 times during the culture period, compared to a 9-fold expansion in the Ihh+/+Rag-/- FTOC (Fig. 4c). This difference was statistically significant (p=0.03). Thus, Ihh promoted thymocyte development before pre-TCR signaling, but negatively regulated thymocyte development after pre-TCR signaling has taken place.

Ihh regulates fetal thymus homeostasis

We have shown that reduced concentration of Ihh promotes differentiation from DN3 to DP stage and that the DN3 subset is the target population for this negative regulatory function of Ihh (Fig. 3a-f, 4a-c). In addition, our expression analysis has shown that thymocytes, and in particular the DP population, provide most of the Ihh in the thymus (Fig. 1a). Concentration of Ihh in the thymus will therefore depend largely on the number of DP thymocytes (ie. Ihh-producing cells). Taken together, our data suggests that Ihh produced by the thymocytes feeds back to negatively regulate the proliferation and differentiation of DN3 cells after pre-TCR signal transduction, in a concentration

dependent manner. Thus, Ihh signaling in the thymus provides a counting system for post-DN3 thymocyte number.

In the heterozygote fetal thymus, the Ihh+/- thymocytes make half the concentration of *ihh* transcript (Fig. 3e) compared to that of their WT counterparts, and thus the amount of Ihh signaling would be approximately half that observed in the WT thymus. Therefore, given this reduced negative regulatory signal in the Ihh+/- thymus, the target cells will proliferate and differentiate faster. This would result in a larger DP population, and thus a larger thymus in total (as observed on E16.5, Fig. 3a-e). Once the size of the DP population increases two-fold, Ihh concentrations will reach WT levels, and so the negative regulatory signal will be fully restored, resulting in a slow-down of proliferation and differentiation. As a consequence DP production would be reduced which would then reduce Ihh signal, and the homeostatic cycle would begin again, leading to increased variation in thymocyte number in the heterozygotes.

This model would therefore predict that on E17.5, the upper limit of size in the Ihh+/- thymus would be greater than in the WT, and also that size would be more varied. To test this, we investigated thymocyte differentiation and proliferation on E17.5. As predicted, the Ihh+/- thymi were on average larger, the upper limits of thymus growth were increased two-fold, and thymocyte number was much more variable ($p<0.0001$) than in the WT thymi (Fig 4d-e). The proportion of DP cells in the heterozygote thymus also mirrored the increased thymocyte number, with larger thymi containing more DP thymocytes (Fig. 4d).

As these data were consistent with concentration dependent negative feedback on DP cell production, we assessed cell cycle status of the target cell population of Ihh's negative

regulatory action (CD25+DN thymocytes, Fig. 4a), from E17.5 Ihh+/- thymi, grouped according to size. PI staining was carried out on purified CD25+ DN thymocytes from E17.5 Ihh+/-, from three groups, I (thymus size > 1.3 relative to WT), II (thymus size 0.9-1.1 relative to WT) and III (thymus size <0.9 relative to WT) (Fig. 4f). In the larger Ihh+/- thymi (I), consistent with Ihh concentration being equivalent to that in WT, the percentage of cells in cycle in the CD25+DN population was the same as that found in WT littermates. In contrast, in the smaller Ihh+/- thymi (III), where Ihh concentrations are lower than WT levels, the percentage of cells in cycle in the CD25+DN population was higher. These data are consistent with the overall concentration of Ihh protein in the thymus influencing proliferation of the CD25+ DN cell, and with negative feed-back restricting thymocyte number by restricting proliferation of an earlier target progenitor cell.

Analysis of E17.5 Ihh-/- thymi revealed partial recovery of thymocyte number and the DP population (Fig. 4d-e), compatible with the positive regulatory function of Ihh being required early, before pre-TCR signal transduction.

Distinct and redundant functions for Ihh and Shh on E16.5

To assess redundancy and overlapping functions of Ihh and Shh on the production of DP cells, we studied the effect of removal of one copy of *ihh* from the E16.5 Shh-/- thymus and vice versa. Removal of one copy of *ihh* from Shh-/- (Shh-/- Ihh+/-) increased cell number three-fold relative to Shh-/- littermate thymi (Fig. 5a), indicating that the negative regulatory function of Ihh (revealed by reducing Ihh concentration) acts on a later stage of T-cell development than the positive Shh signal, so that reduction of Ihh signal allowed partial recovery of thymocyte number in the Shh-/- thymus. In the Shh+/- Ihh+/-

thymus, thymocyte number was not greater than in the Shh+/+Ihh+/-, but was increased relative to WT, and there was greater variability. As shown previously[5], the proportion of DP cells was reduced in Ihh-/- and in Shh-/- thymi, and there was an increase in the proportion of DN cells, Fig. 3c-f, 4d). Deletion of one copy of *shh* in Ihh-/- thymi (Shh+/-Ihh-/-) caused a greater reduction in the proportion of DP cells, than seen in Ihh-/- thymi, in all litters examined (Fig 5b-c). The proportion of DP cells was 3.6% in the Shh+/-Ihh-/-, compared to 19.7% in the Shh+/-Ihh+/+ and 12% in the Shh+/+Ihh-/- littermate thymi (Fig 5c). Thus, both Ihh and Shh provide positive signals for DN cell expansion and differentiation, and it is the total amount of Hh signal that is critical at this stage.

The fact that the number of thymocytes was increased in the Ihh+/-Shh-/- thymus compared to Ihh+/+Shh-/- littermates, allows a clear distinction to be made between the positive and negative regulatory roles for Hh signaling and indicates that Ihh concentration is important after Shh signaling, as rather than aggravating the phenotype, lowering the concentration of Ihh actually allowed partial recovery of DP thymocyte number.

Concentration dependent regulation of thymocyte development by recombinant-Hedgehog (r-Hh) treatment in FTOC

To ask if we could reconsitute the Ihh-/- and Ihh+/- thymus with exogenous Hedgehog protein we treated FTOC with r-Hh. Mammalian Hh proteins are autocatalytically processed to form an active N-terminal fragment which is highly homologous between Shh and Ihh and between species[6,7]. We therefore treated FTOC with recombinant mouse Shh N-terminus (r-mShh-N). We have previously shown that high dose treatment of FTOC with octylated recombinant human Shh N-terminus (oct-r-hShh-N) arrested

thymocyte development at the DN stage[5,10], whereas treatment with low concentrations of this protein increased thymocyte production[5]. We therefore titrated the r-mShh-N in WT FTOC and measured DP cell production after three days (Fig. 5d). The activity of the r-mShh-N was very similar in FTOC to that of the oct-r-hShh-N we had used in previous studies, and at 1.5μg/ml r-mShh-N arrested thymocyte development at the DN stage, decreasing the number of DP cells 80-fold, whereas at the lowest concentration we assayed (0.05μg/ml), we saw a modest increase in the production of DP cells (Fig. 5d).

We then asked if treatment of Ihh+/-FTOC with r-Hh could restore the negative regulatory effect of Ihh on thymocyte development. As Ihh+/- thymi contain approximately twice the number of thymocytes as WT thymi on E16.5, we chose a concentration of r-Hh that decreased DP production approximately two-fold in our titration (Fig. 5d). In FTOC, we treated E15 Ihh+/- thymus lobes for 3 days with 0.25μg/ml r-mShh-N and compared thymocyte development to that in the untreated lobe from the same thymus and to WT littermate thymus lobes. The untreated Ihh+/- FTOC produced approximately four-times more thymocytes than their WT counterparts, and this expansion was evident in DN, CD8ISP and DP populations (Fig. 5e-f). Treatment with 0.25μg/ml r-mShh-N restored the negative regulatory signal and inhibited thymocyte development to WT levels. A reduction was seen not only in the number of DP cells produced, but also in the expansion of the DN population (Fig. 5e-f).

We then asked if we could restore the positive regulatory function of Ihh on thymocyte development by treatment of Ihh-/-FTOC with r-Hh. Given that treatment of Ihh+/-FTOC with 0.25μg/ml r-mShh-N restored the heterozygote thymus to WT thymocyte production, we reasoned simplistically that treatment of the Ihh-/- thymus with the same

concentration of r-mShh-N should mirror the Ihh+/- thymus, in which Ihh protein concentrations are sufficient to provide the early positive regulatory signal for thymocyte development but not the later negative regulatory signal. We treated Ihh-/- and littermate FTOC with 0.25μg/ml r-mShh-N and compared thymocyte development in the treated thymus lobes with that in the untreated lobe from the same thymus. As seen previously (Fig. 5d-f), r-mShh-N inhibited thymocyte development in the littermate FTOC, but as predicted by our model, the treatment promoted thymocyte development in the Ihh-/- with increases in DN, ISP and DP production (Fig. 5g). Thus, treatment with r-Hh had opposing outcomes on Ihh-/- and Ihh+/- FTOC, and we demonstrated both the positive and negative regulatory functions of Ihh *in vitro*.

Ihh signaling in the adult heterozygote thymus

To ask if Ihh is important in the adult thymus, we confirmed *ihh* expression in sorted adult thymocyte populations, by quantitative RT-PCR. We detected *ihh* transcription in all thymocyte populations, with a six-fold upregulation between the DN3 and DP populations (Fig. 6a). Given that the DP population makes up more than 80% of adult thymocytes, and expresses more *ihh* than other thymocyte populations or the stroma, it produces most of the Ihh protein in the thymus. We also assessed *gli1* expression in sorted adult populations, and as reported previously[21,22], the pattern of *gli1* transcription in adult thymocytes was similar to that in fetal thymocytes, with expression peaking in the CD25+ (DN2/DN3) DN cells, and virtually undetectable in the DP population (Fig. 6b), indicating that adult DP cells, although the major producers of Ihh, are not themselves responding to a Hh signal[10,21].

To ask if Ihh is also a negative regulator of adult thymus homeostasis, we compared the thymus of adult inbred Ihh+/- and WT littermates. As seen in the fetal heterozygote thymus (Fig 3a, 4e), the upper limit to Ihh+/- thymocyte number was approximately two fold greater than in the WT littermates (Fig 6c), there was more variation between individual thymi, and *ihh* transcription in Ihh+/- DP cells was one half that in Ihh+/+ DP cells (Fig. 6d), consistent with negative feedback of Ihh from the DP population on their DN progenitors.

Ihh signaling in the adult Ihh conditional knockout thymus

To further study the role of Ihh produced by the thymocytes, rather than the epithelium, on T-cell development in the adult thymus, we used two different conditional ('floxed') Ihh mouse models[15]. Excision was mediated by transgenic Cre in either the vav or CD2 transgenic cassettes[27]. The vav cassette drives Cre expression in all haematopoietic cells, including thymocytes at all stages of their development, but not thymic epithelium. The CD2 transgenic cassette drives Cre expression in lymphocytes only, with partial expression in thymocytes starting at the DN2 stage, and complete expression in all thymocytes only from the DN4 stage onwards[27].

In the case of the vavCre model, we confirmed efficient excision of the floxed *ihh* allele by PCR amplification of the *ihh* gene from thymocyte DNA from vavCre- Ihhfl/fl and vavCre+Ihhfl/fl littermates (Fig. 6e). We compared mice either in which both *ihh* alleles were floxed and excised by vavCre expression (vavCre+Ihhfl/fl), or in which one allele was null and the other floxed and excised (vavCre+Ihhfl/-), and found a significant reduction in thymocyte number in both cases (Fig. 6f-g). To demonstrate functionally that Ihh produced by thymocytes can mediate the early positive signal for differentiation,

we assessed the DN populations in vavCre-Ihhfl/fl (littermate) and vavCre+Ihhfl/fl littermates (in which both alleles of *ihh* are present in the thymus epithelium). In the vavcre+Ihhfl/fl thymus where Cre expression is complete at the DN1 stage[27], there was a significant reduction in the DN4 population (from 39.4% to 20.1%), and a concomitant increase in the proportion of DN3 cells (from 41.4% to 57.7%) (Fig. 6h-i). As seen in the fetal Ihh-/- thymus, a slightly higher proportion of the DN3 population expressed icTCRβ than in the corresponding littermate DN3 population (Fig. 6j-k), indicating that TCRβ locus rearrangement was not limiting DP cell production.

For the CD2Cre model, we confirmed efficient excision of the floxed *ihh* allele by PCR amplification of the *ihh* gene from thymocyte DNA from CD2Cre- Ihhfl/fl and CD2Cre+Ihhfl/fl littermates (Fig. 7a). Analysis of the CD2Cre+Ihhfl/fl mice showed that on average thymi were larger and the upper limit of thymocyte number was increased relative to littermates (Fig 7b), indicating loss of negative regulation. However, there was no significant difference in the proportion of DN3 and DN4 cells (Fig. 7c-d), indicating that the earlier positive signal was not affected. Thus, in the CD2cre+Ihhfl/fl mice, where excision of Ihh happens at a later developmental stage than in the vavCre+Ihhfl/flmice, Ihh's positive regulatory role before pre-TCR signaling remains intact, whereas its later negative regulatory role is compromised. The fact that, in contrast to the vavCre+Ihhfl/flmice, DN populations were not effected in the CD2Cre+Ihhfl/fl is consistent with the later excision described in the CD2Cre model[27], and indicates that Ihh production by the early DN populations provides an autocrine signal. Both conditional knock out models therefore demonstrate the functional importance of Ihh secreted by the thymocytes themselves. In both cases some Ihh will

still be produced by the thymic epithelium, but this is insufficient to provide either the full early positive signals, or subsequent negative regulatory signals that control thymus homeostasis, hence the phenotypes observed.

Discussion

Here we show that the secreted signaling molecule Ihh regulates T-cell development, influencing thymus homeostasis and thymocyte number in both adult and fetus. Mice mutant in genes encoding several other secreted signaling molecules have reduced thymocyte numbers[4,5], but the phenotype of Ihh mutant mice is unusual in that although thymocyte number and the transition to DP cell were reduced in the Ihh-/- thymus, they were actually increased in the Ihh+/- thymus, suggesting a novel role for Ihh in thymocyte homeostasis.

Analysis of both heterozygote and conditional Ihh knockout models showed that expression of Ihh by thymocytes is required to restrict the size of the thymus by transmitting negative signals to limit thymocyte number, and in vitro treatment of Ihh+/- FTOC with r-Hh protein restored this negative regulatory function.

The fact that Ihh was most highly expressed by the DP population which have down-regulated both *gli1* and *smo* transcription[10,21,22] and are not Hh-responsive[10], suggests that Ihh regulates thymus homeostasis by providing a negative feed-back loop on the production of DP cells (Fig. 7c). Analysis of proliferation of the different thymocyte subsets indicated that this feedback targets the DN3 population. In addition, analysis of vavCre+Ihhfl/fl and CD2Cre+Ihhfl/fl thymi, and of Ihh+/-Rag-/- FTOC, showed that Ihh provides a positive signal for differentiation before pre-TCR signal transduction, and a negative signal after pre-TCR signal transduction. Hh signaling is necessary for efficient generation of DP cells, as evidenced by the reduction in DP cells in the Shh-/-, Ihh-/- and Ihh-/-Shh+/- thymi**,** and by the increase in DP production *in vitro* by treatment of Ihh-/- FTOC with exogenous r-Hh. Both Shh made by the thymus epithelium[5,10,20], and Ihh

made by thymocytes and epithelium, promote early thymocyte differentiation before pre-TCR signal transduction. However, as the production of DP and SP cells increases (both of which express higher levels of *ihh* than their DN progenitors), so would the concentration of Ihh, which could then, having reached a critical threshold, provide negative feedback on the DN population, preventing the thymus growing *ad infinitum*. In the Ihh+/- thymus, the concentration of Ihh produced would be one-half of the physiological level produced in the WT thymus, allowing the thymus to grow approximately two times larger, before becoming subject to the 'normal' (WT) levels of negative regulatory feedback. Analysis of the E16.5 fetal thymus facilitated detection of this negative feedback, as the first wave of production of DP thymocytes is synchronized. Studies of conditional Smo knockout thymi showed that Hh pathway activation in thymocytes is essential for their survival, proliferation and differentiation at the transition from DN1 to DN2[21]. Our analysis of double mutants indicated that although Shh secreted by the epithelium is dominant in providing this signal, Ihh, produced by thymocytes and stroma, has a redundant function to promote differentiation at the transition from DN1 to DN2. In addition, the double mutant analysis on E16.5 enabled a clear distinction between the positive and negative regulatory functions to be made, as removal of one copy of *ihh* from the Shh-/- alleviated, rather than aggravated, the Shh-/- phenotype and increased the production of DP cells, indicating that the negative regulatory action of Ihh is at a later developmental stage than the positive function of Shh. These different requirements for Shh and Ihh may also be due to differences in concentrations of the two proteins. They are also likely to be due to the different spatial

and temporal expression patterns of Shh and Ihh[10,20], and are consistent with Ihh being expressed most highly by DP cells, after Shh is required.

The phenotype of the Ihh+/- and Ihh-/- mice provides an explanation for an apparent discrepancy between data obtained from *in vitro* experiments[10] and from the *ex vivo* analysis of Shh-/-[5] and conditional Smo knock-out thymi[21]. In FTOC, partial removal of endogenous Hh activity by treatment with neutralizing anti-Hh antibody (that can bind both Shh and Ihh) increased differentiation from DN to DP cell[10]. Likewise, the development of human CD34+ thymocyte progenitors *in vitro* was accelerated by neutralization of Hh signaling[24]. These observations seemed to conflict with the phenotype of the Shh-/- thymus, which showed reduced differentiation from DN to DP cell[5]. Our analysis of Ihh mutant embryos demonstrates that *in vivo* T-cell development is regulated by the overall concentration of Hh protein that the developing thymocyte receives, and that in WT thymi, Ihh protein can actually function as a brake on DP cell production (explaining the impact of the reduction in Hh signaling by treatment with neutralizing anti-Hh antibody in WT FTOC). Clearly, the concentration of Ihh protein that a given thymocyte receives will depend on the architecture of the thymus and the position of that cell in the thymus, relative to Ihh-producing cells.

Conditional deletion of Smo from T-lineage cells, using transgenic Cre under the control of the lck promotor[21], did not show an influence of Hh signaling on T-cell development after the DN2 stage, whereas the data presented here demonstrate that Ihh regulates the rate of differentiation from DN3 to DP cell. It would not be possible to detect the negative regulatory function of Ihh on the DN to DP transition in the conditional Smo knockout, as Smo-deficient DN3 population would be unable to transduce any Ihh signal.

Consistent with our model, in which Ihh production by DP cells feeds back to signal to the DN population, deletion of Smo using transgenic Cre controlled by the CD4 promoter had no effect on T-cell development, confirming that the DP cells are not themselves responding to the Ihh they secrete[21].

The fact that Hh pathway activation in thymocytes increases differentiation and expansion at the earliest stages of their development but reduces differentiation and proliferation at later developmental stages might seem surprising, but has parallels in the development of other tissues, such as gut and retina[32,33]. For example, in retinal development, Hh signaling has different effects at different stages of development, and has been shown to push precursor cells out of the cell cycle, to signal for differentiation at a distinct stage, and can also promote proliferation[32,34-36]. A cell's interpretation of the Hh signal will depend on many factors, including strength and duration of signal, and the external and intra-cellular context of signal transduction[7,16,37], and so Hh signaling may effect stem, progenitor and mature cells differently, and have opposing effects on cellular processes, depending on the state of differentiation of the cell.

In the future it will be important to identify the molecular mechanisms and transcriptional targets that account for the positive and negative regulatory functions of Hh signaling on thymocyte development. Both cell cycle inhibitors, such as cdkn1 and cdkn2 family molecules, and factors required for cell cycle progression, such as cyclin D2 are Hh target genes in other cell types[32,36,38,39]. It is therefore possible that cell cycle inhibitors are transcriptional targets of the Hh pathway accounting for the negative regulatory function, whereas cyclin D2 is a Hh target in the DN2 population, accounting for the earlier positive regulatory function. The Hedgehog signaling pathway interacts with BMP and

Wnt signaling pathways in the development of other tissues[19,40]. BMP2/4 signalling has previously been shown to negatively regulate thymocyte development,[41-43] but Wnt signaling promotes thymocyte development[4]. It is therefore possible that the opposing positive and negative functions of Ihh in thymocyte development are mediated in part by Wnt and BMP2/4 signaling respectively.

In summary, we show that Ihh produced by thymocytes promotes T-cell development before pre-TCR signal transduction, and limits T-cell development after pre-TCR signal transduction in a concentration dependent manner, thereby restricting thymocyte production and thymus size.

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Authorship and Conflict of Interest Statement

All authors contributed experimentally to the manuscript. SO, A H-T and DS are the equal and major contributors of the data described in this manuscript and * shown on the title page denotes this equal first author contribution.

SO, A H-T, DS and TC prepared the written manuscript. There is no conflict of interest.

Figure legends

Figure 1: Early thymocyte development in Ihh-/- thymi.

(A and B) Transcription of *ihh* (**A**) and *gli1* (**B**) in sorted E16.5 fetal thymocyte populations and thymus stroma from C57BL/6 mice. Levels of *ihh* and *gli1* transcription were normalized for *HPRT* mRNA content and are shown relative to *HPRT* normalized transcription in the DN3 (*ihh*) or DN1 (*gli1*) subsets. cDNA samples were analyzed in triplicate by quantitative PCR on an iCycler (Bio-Rad Laboratories) using iQ-SYBR Green Supermix (Bio-Rad). Thymocytes were sorted on a Modular Flow Cytometer (MoFlo, Cytomation), and purity was >98%.

(C to F) Flow cytometry of E13.5 Ihh-/- and WT littermate thymi. **(C)**: Dot plot of anti-CD45.2 staining versus FSC, showing the gate used for analysis of lymphocyte precursors. **(D)**: The composition of DN subsets gated on CD45.2+ and stained with anti-CD44 and anti-CD25. **(E):** Dot plot of anti-CD117 staining versus anti-CD44 staining, gated on CD45.2+. **(F):** Histogram of anti-B220 staining, gated on CD45.2+CD44+ cells. Thymus size: Control: 6.4×10^3, Ihh-/-: 4.8×10^3

(**G**) (**i**) Photographs of E14.5 Shh-/- and WT littermate thymus lobes (**ii**) Photographs of E14.5 Ihh-/- and WT littermate thymus lobes.

(**H**) The mean (± SE) relative cell number in WT (n = 7), Ihh+/- (n = 17) and Ihh-/- (n = 5) thymi on E14.5. To allow comparison between litters, the number of cells recovered from each thymus was divided by the mean number of cells recovered from WT thymi from the same litter, to give relative cell number from four E14.5 litters. Differences between WT, Ihh+/- and Ihh-/- were not significant by students t-test ($p>0.05$).

Figure 2: Redundancy between Shh and Ihh in early thymocyte development.

(A and B) Flow cytometry of E13.5 Ihh+/+, Ihh+/-, Ihh-/-, and Ihh-/-Shh+/- thymi.

(A) Dot plot of anti-CD45.2 staining versus FSC, showing the gate used for analysis of lymphocyte precursors. **(B)** CD44 and CD25 expression of thymocytes, gated on CD45.2 positive cells. Thymus sizes were: Ihh+/+: 4.8 x 10^3, Ihh+/-: 6.4 x 10^3, Ihh-/-: 4.8 x 10^3 and Ihh-/- Shh+/-: 6.0 x 10^3

Figure 3: Thymocyte development in E16.5 Ihh-/- and Ihh+/- thymi.

(**A**) Thymocyte number on E16.5.

The mean relative cell number (± SE) in WT, Ihh+/- and Ihh-/- thymi on E16.5. To allow comparison between litters, the number of cells recovered from each thymus was divided by the mean number of cells recovered from WT thymi from the same litter, to give relative cell number from different E16.5 litters. The difference in mean cell number between Ihh-/- and WT thymi ($p<0.0001$), Ihh+/- and WT thymi ($p=0.018$) and Ihh-/- and Ihh+/- ($p<0.0001$) were statistically significant by Students t-test.

(**B**) Photographs of E16.5 WT, Ihh+/- and Ihh-/- littermate thymus lobes.

(**C**) The mean relative cell number (dark bars) and mean relative percentages (open bars) of DP cells (±SE) in WT, Ihh+/- and Ihh-/- thymi on E16.5. Differences in mean cell number and percentages between WT and Ihh+/- ($p=0.007$, $p=0.02$), WT and Ihh-/- ($p<0.0001$ for both) and Ihh+/- and Ihh-/- ($p<0.0001$ for both) thymi were statistically significant by Students t-test.

(**D**) Flow cytometry of E16.5 WT, +/- and -/- littermate thymi stained with anti-CD4 and anti-CD8. Thymus size: WT 4.56 x 10^4, Ihh+/- 1.144 x 10^5 and Ihh-/- 1.68 x 10^4.

(**E**) Transcription of *ihh* in DP thymocytes from embryonic Ihh+/+ and Ihh+/- thymi. Relative levels of *ihh* transcription were measured as described in Fig. 1a and were normalized for *HPRT* mRNA content.

(F) Left-hand panel: Mean percentages of DN thymocytes in WT and Ihh-/- thymi, relative to mean of WT littermates. The percentage of DN cells in the Ihh-/- thymus is significantly higher than in the Ihh+/+ thymi by Students t-test (p=0.002). Right-hand panel: Mean percentages of DN4 thymocytes in the DN subset relative to mean of WT littermates are shown for WT and Ihh-/- thymi. Differences between Ihh+/+ and Ihh-/- were statistically significant by Students t-test (p=0.0001).

(G) Mean percentages of intracellular (ic)TCRβ+ cells in the DN3 subset are shown relative to mean of WT littermates for WT and Ihh-/- thymi. Differences between mean %icTCRβ+ in DN3 subset of Ihh+/+ and Ihh-/- were statistically significant by Students t-test (p=0.03).

(H) Representative histograms of icTCRβ expression in the DN3 subsets of WT litter mate (top) and Ihh-/- (bottom) thymi.

(I) TCRβ locus rearrangement was measured in E15.5 DN thymocytes from WT and Ihh-/- littermates, according to the method of Gounari et al [29]. DNA was amplified using primers 5' to Vβ8.2 or Vβ5.1 and 3' to Jβ2.7 and products were measured by quantitative PCR on an iCycler (Bio-Rad Laboratories) using iQ-SYBR Green Supermix (Bio-Rad), DNA content was normalized relative to *Thy1*. Differences between Ihh-/- and WT were not significant by students t-test.

(J) The bar chart shows the % of cells in S+G2/M, measured by PI staining of FACS sorted E16.5 CD25+ DN cells from WT and Ihh-/- littermates, relative to the mean % in

WT littermate populations. Differences in mean % between -/- and WT were not statistically significant

Figure 4: Ihh regulates thymus homeostasis

(A) Scatter plots show the % of cells in S+G2/M, measured by PI staining of FACS sorted E16.5 CD25+ DN (left-hand plot), CD8ISP (middle plot) and DP (right-hand plot) populations from WT and Ihh+/- littermates, relative to the mean % in WT littermate populations. Differences in mean % between +/- and WT were statistically significant for CD25+DN (p=0.042) and CD8ISP (p= p=0.0401) populations, but not for DP cells (p=0.981).

(B) Scatter plots show relative thymocyte number from Ihh+/+Rag1-/- and Ihh+/-Rag1-/- thymi (relative to mean of Ihh+/+Rag1-/- littermates). Differences are significant by students t-test (p=0.0013). Mean thymocyte number: Litter 1 Ihh+/+Rag1-/- $1.65x10^6$, Ihh+/-Rag1-/- $1.33x10^6$; Litter 2 Ihh+/+Rag1-/- $1.7x10^6$, Ihh+/-Rag1-/- $1.25x10^6$; Litter 3 Ihh+/+Rag1-/- $1.75x10^6$, Ihh+/-Rag1-/- $1.31x10^6$.

(C) Bar chart shows fold increase in thymocyte number on induction of differentiation by treatment with 1μg/ml anti-CD3 after 5 days FTOC for Rag1-/-Ihh+/+ and Rag1-/- Ihh+/- littermates. For each thymus, one lobe was cultured with anti-CD3 and the fold-increase in cell number was calculated relative to the number of cells in the other untreated lobe. The fold-increase in the Rag1-/-Ihh+/- was significantly different from that in the Rag1-/-Ihh+/+ by students t-test (p= 0.03). Mean thymocyte number: Litter 1 Ihh+/+Rag1-/- untreated $1.65x10^6$, Ihh+/+Rag1-/- +anti-CD3 $1.425x10^7$, Ihh+/-Rag1-/- untreated $1.33x10^6$, Ihh+/-Rag1-/- +anti-CD3 $1.725x10^7$. Litter 2 Ihh+/+Rag1-/- untreated 1.7

$x10^6$, Ihh+/+Rag1-/- +anti-CD3 $1.49x10^7$, Ihh+/-Rag1-/- untreated $1.245x10^6$, Ihh+/+Rag1-/- +anti-CD3 $1.689x10^7$.

(D) Dot plots show E17.5 WT, Ihh+/- and Ihh-/- thymi stained with anti-CD4 and anti-CD8. Mean thymocyte number: Litter 1 WT $2.05x10^6$, Ihh+/- $3.3x10^6$, Ihh-/-$2x10^6$; Litter 2 WT $3.5x10^6$, Ihh+/- $2.865x10^6$, Ihh-/- $8.5x10^5$; Litter 3 WT $3.35x10^6$, Ihh+/- $2.63x10^6$ Ihh-/-$1.675x10^6$; Litter 4 WT $3.35x10^6$, Ihh+/- $2.336x10^6$; Litter 5 WT $3.868x10^6$, Ihh+/- $3.69x10^6$; Litter 6 WT $4.8x10^6$, Ihh+/- 4.756, Ihh-/- $3.8x10^6$.

(**E**) Scatter plot shows cell numbers (relative to mean of WT) from thymi from E17.5 WT, Ihh+/- and Ihh-/- embryos. The difference in mean between WT and Ihh-/- is significant ($p=0.0018$, students t-test) and the difference in standard deviation between WT and Ihh+/- is also significant ($p< 0.0001$, F-test)

(**F**) Scatter plot shows **(i) filled diamonds:** thymocyte number (relative to mean of WT) from E17.5 WT littermate and Ihh+/- embryos. Ihh+/- thymi are grouped by relative size into three sets: I >1.3, II = 0.9-1.1 and III <0.9; (ii) **open circles:** the % of cells in S+G2/M (relative to mean of WT), measured by PI staining of Facs sorted E17.5 CD25+ from WT and Ihh+/- mice grouped into three groups I, II and III as above.

Figure 5: Thymocyte development in Shh/Ihh double mutants and in FTOC treated with exogenous recombinant Hedgehog protein

(A) Histograms show the mean relative cell number (± SE) in WT (n = 11), Ihh+/+Shh+/- (n = 5), Ihh+/-Shh+/+ (n = 8), Ihh+/- Shh+/- (n= 3), Ihh-/-Shh+/+ (n = 4), Ihh-/-Shh+/- (n = 3) (left) and in WT (n=11), Ihh+/+Shh-/- (n = 3) and Ihh+/-Shh-/- (n = 3) thymi (right) on E16.5.

(**B**) Histogram to show the mean relative percentage (± SE) of DP cells in the litters shown in (A).

(**C**) Expression of CD4 and CD8 on Shh+/- Ihh+/+, Shh+/+ Ihh-/- and Shh+/- Ihh-/- thymi. Thymus sizes were: Shh+/- Ihh+/+: 2.6 x 10^5, Shh+/+ Ihh-/-: 1.28 x 10^5 and Shh+/- Ihh-/-: 5.2 x 10^4.

(**D**) Bar chart shows the fold change in number of DP thymocytes recovered from WT E15 FTOC treated for 3 days with different concentrations of r-mShh-N (R&D Systems), compared to the number of DP cells recovered from the untreated thymus lobe from the same embryos. 0.25μg/ml r-mShh-N decreased the production of DP cells by approximately two-fold.

(**E**) Bar chart shows the number of cells from DN, CD8+ISP and DP thymocyte populations recovered from Ihh+/- and WT littermate E15 FTOC cultured for three days, with or without treatment with 0.25mg/ml r-mShh-N. Mean cell recovery per thymus lobe : Ihh+/- control 3.3 x 10^5; Ihh+/- + r-mShh-N 6.3 x 10^4 ; Ihh+/+ littermate control 7.0 x 10^4. In each experiment, the treated thymus lobe was compared to the untreated lobe from the same embryo.

(**F**) Dot plots show anti-CD4 and anti-CD8 staining of individual thymus lobes from the experiment in (E), in which treated thymus lobes were compared to the untreated lobe form the same embryo, or to a WT littermate lobe.

(**G**) Bar charts show the number of cells from DN, CD8+ISP and DP thymocyte populations recovered from Ihh-/- and littermate (LM) E15 FTOC cultured for five days, with or without treatment with 0.25μg/ml r-mShh-N. The treated lobe from one embryo was compared to the untreated lobe from the same embryo.

Figure 6: Thymocyte Ihh expression is required for adult thymocyte development.

(A-B) Expression of Ihh (A) and Gli1 (B) was assessed by QRT-PCR in FACS sorted populations of adult and fetal murine tissue. Levels of *ihh* and *gli1* transcription were normalized for *HPRT* mRNA content and are shown relative to *HPRT* normalized transcription in the CD25+DN population (*ihh*) and DN1 population (*gli1*). Thymocytes were sorted on a Modular Flow Cytometer (MoFlo, Cytomation), and purity was >98%.

(C) Relative thymocyte number (relative to the mean of WT littermates) of three week old WT (n=8) and Ihh+/- (n=8) littermates, revealed a significant difference in standard deviation as judged by F-test (p=0.0009). Mean thymocyte number: Litter 1 WT $1.2x10^8$, Ihh+/- $1.77x10^8$; Litter 2 WT $1.44x10^8$, Ihh+/- $1.76x10^8$; Litter 3 WT $1.35x10^8$, Ihh+/- $1.81x10^8$.

(D) Transcription of *ihh* in DP thymocytes from Ihh+/+ and Ihh+/- thymi. Relative levels of *ihh* transcription were measured as described in Fig. 1a and were normalized for *HPRT* mRNA content.

(E) Excision of the *ihh* gene as assessed by PCR from vavCre- (lane1) and vacre+ Ihhfl/fl mice (lane 2).

(F) Relative cell number (calculated relative to the mean of the WT littermates) of the thymus of 4 to 6 week old mice of littermates (all Cre- : non-knockout mice, n=9) compared to knockout mice (vavCre+ Ihhfl/null, n=4) showed a significant decrease in thymocyte cell number (p=0.00284). Mean thymocyte number: Litter 1 Littermate (LM) $1.7x10^8$, vavcre+Ihhfl/null $0.75x10^8$; Litter 2 LM $1.95x10^8$, vavcre+Ihhfl/null

$0.47x10^8$; Litter 3 LM $1.48x10^8$, vavcre+Ihhfl/null $1.28x10^8$; Litter 4 LM $1.2x10^8$, vavcre+Ihhfl/null 9.2×10^7.

(G) Relative cell number (calculated as in D) of the thymus isolated from 4 to 6 week old mice of littermate vavCre- Ihhfl/fl mice (wildtype, n=15) compared with vavCre+ Ihhfl/fl mice (knockout mice, n=5) showed a significant decrease in thymocyte number (p=0.0158). Mean thymocyte numbers: Litter 1 vavCre- Ihhfl/fl $3x10^8$, vavCre+ Ihhfl/fl $1.925x10^8$; Litter 2 vavCre- Ihhfl/fl $3.24x10^8$, vavCre+ Ihhfl/fl $2.59x10^8$; Litter 3 vavCre- Ihhfl/fl $2.115x10^8$, vavCre+ Ihhfl/fl $1.3x10^8$; Litter 4 vavCre- Ihhfl/fl $2.8x10^8$, vavCre+ Ihhfl/fl $1.75x10^8$.

(H) FACS analysis of CD44 and CD25 expression on CD4-8-3- thymocytes from vavCre- Ihhfl/fl and vavCre+ Ihhfl/fl mice revealed reduced transition to the DN4 stage in development.

(I) Histogram shows the percentage of DN4 cells in the DN population, relative to the mean percentage in Cre- littermates for vavCre- Ihhfl/fl (n=13) and vavCre+ Ihhfl/fl knockout mice (n=6). The differences were significant by students t-test (denoted by *) (p =0.028).

(j) Intracellular TCRβ expression was analyzed in DN3 thymocytes from littermate vavCre- Ihhfl/fl WT (n=14) and vavCre+ Ihhfl/fl knockout mice (n=6).

(k) The bar chart shows the percentage of icTCRβ+ cells in the DN3 population, relative to the mean percentage of icTCRβ+ cells in the DN3 population in WT littermates, for vavCre- Ihhfl/fl WT (n=14) and vavCre+ ihh fl/fl knockout mice (n=6). A significant increase in icTCRβ expression was observed in vavCre+ Ihhfl/fl knockout DN3 population, as determined by students t-test (p=0.0122).

Figure 7: Ihh produced by thymocytes signals to negatively regulate differentiation from DN to DP in the adult.

(A) Excision of the *ihh* gene as assessed by PCR from CD2Cre- (lane 1) and CD2Cre+ Ihhfl/fl (lanes 2) thymocytes.

(B) Relative thymocyte number (relative to the mean of WT littermates) of 4 to 6 week old CD2Cre- and vavCre+Ihhfl/fl littermates showed a significant difference in standard deviation, by F-test (p=0.0001). Mean Thymocyte number: Litter 1 CD2Cre- Ihhfl/fl 3.77 $x10^8$, CD2Cre+Ihhfl/fl 4.225$x10^8$; Litter 2 CD2Cre- Ihhfl/fl 1.03$x10^8$, CD2Cre+Ihhfl/fl 1.245$x10^8$ **;** Litter 3 CD2Cre- Ihhfl/fl 2.84$x10^8$, CD2Cre+Ihhfl/fl 3.575$x10^8$; Litter 4 CD2Cre-Ihhfl/fl 1.08$x10^8$, CD2Cre+Ihhfl/fl1.34$x10^8$; Litter 5 CD2Cre-Ihhfl/fl 2.67$x10^8$, CD2Cre+Ihhfl/fl 3.31$x10^8$.

(C) FACS analysis of CD44 and CD25 expression on CD4-8-3- thymocytes from CD2Cre- Ihhfl/fl and CD2Cre+ Ihhfl/fl mice.

(D) Histogram shows the percentage of DN4 cells in the DN population, relative to the mean percentage in Cre- littermates for CD2Cre- Ihhfl/fl (n=13) and CD2Cre+ Ihhfl/fl knockout mice (n=6). The differences were not significant by students t-test.

(E) Model of functions of Shh and Ihh signaling in the control of thymocyte homeostasis.

References

1. DiSanto JP, Muller W, Guy-Grand D, Fischer A, Rajewsky K. Lymphoid development in mice with a targeted deletion of the interleukin 2 receptor gamma chain. Proc Natl Acad Sci U S A. 1995;92:377-381.

2. Radtke F, Wilson A, Stark G, et al. Deficient T cell fate specification in mice with an induced inactivation of Notch1. Immunity. 1999;10:547-558.
3. Maillard I, Fang T, Pear WS. Regulation of lymphoid development, differentiation, and function by the Notch pathway. Annu Rev Immunol. 2005;23:945-974.
4. Staal FJ, Clevers HC. Wnt signaling in the thymus. Curr Opin Immunol. 2003;15:204-208.
5. Shah DK, Hager-Theodorides AL, Outram SV, Ross SE, Varas A, Crompton T. Reduced thymocyte development in sonic hedgehog knockout embryos. J Immunol. 2004;172:2296-2306.
6. Ingham PW, McMahon AP. Hedgehog signaling in animal development: paradigms and principles. Genes Dev. 2001;15:3059-3087.
7. Ingham PW, Placzek M. Orchestrating ontogenesis: variations on a theme by sonic hedgehog. Nat Rev Genet. 2006;7:841-850.
8. Varas A, Hernandez-Lopez C, Valencia J, et al. Survival and function of human thymic dendritic cells are dependent on autocrine Hedgehog signaling. J Leukoc Biol. 2008;83:1476-1483.
9. Uhmann A, Dittmann K, Nitzki F, et al. The Hedgehog receptor Patched controls lymphoid lineage commitment. Blood. 2007;110:1814-1823.
10. Outram SV, Varas A, Pepicelli CV, Crompton T. Hedgehog signaling regulates differentiation from double-negative to double-positive thymocyte. Immunity. 2000;13:187-197.
11. Bhardwaj G, Murdoch B, Wu D, et al. Sonic hedgehog induces the proliferation of primitive human hematopoietic cells via BMP regulation. Nat Immunol. 2001;2:172-180.
12. Chiang C, Litingtung Y, Lee E, et al. Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. Nature. 1996;383:407-413.
13. St-Jacques B, Hammerschmidt M, McMahon AP. Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation. Genes Dev. 1999;13:2072-2086.
14. Zhang XM, Ramalho-Santos M, McMahon AP. Smoothened mutants reveal redundant roles for Shh and Ihh signaling including regulation of L/R symmetry by the mouse node. Cell. 2001;106:781-792.
15. Razzaque MS, Soegiarto DW, Chang D, Long F, Lanske B. Conditional deletion of Indian hedgehog from collagen type 2alpha1-expressing cells results in abnormal endochondral bone formation. J Pathol. 2005;207:453-461.
16. Ashe HL, Briscoe J. The interpretation of morphogen gradients. Development. 2006;133:385-394.
17. Borowski C, Martin C, Gounari F, et al. On the brink of becoming a T cell. Curr Opin Immunol. 2002;14:200-206.
18. Petrie HT, Tourigny M, Burtrum DB, Livak F. Precursor thymocyte proliferation and differentiation are controlled by signals unrelated to the pre-TCR. J Immunol. 2000;165:3094-3098.
19. Crompton T, Outram SV, Hager-Theodorides AL. Sonic hedgehog signalling in T-cell development and activation. Nat Rev Immunol. 2007;7:726-735.

20. Sacedon R, Varas A, Hernandez-Lopez C, et al. Expression of hedgehog proteins in the human thymus. J Histochem Cytochem. 2003;51:1557-1566.
21. Andaloussi AE, Graves S, Meng F, Mandal M, Mashayekhi M, Aifantis I. Hedgehog signaling controls thymocyte progenitor homeostasis and differentiation in the thymus. Nat Immunol. 2006;7:418-426.
22. Rowbotham NJ, Hager-Theodorides AL, Cebecauer M, et al. Activation of the Hedgehog signaling pathway in T-lineage cells inhibits TCR repertoire selection in the thymus and peripheral T-cell activation. Blood. 2007;109:3757-3766.
23. Hager-Theodorides AL, Dessens JT, Outram SV, Crompton T. The transcription factor Gli3 regulates differentiation of fetal CD4- CD8- double-negative thymocytes. Blood. 2005;106:1296-1304.
24. Gutierrez-Frias C, Sacedon R, Hernandez-Lopez C, et al. Sonic hedgehog regulates early human thymocyte differentiation by counteracting the IL-7-induced development of CD34+ precursor cells. J Immunol. 2004;173:5046-5053.
25. Rowbotham NJ, Furmanski AL, Hager-Theodorides AL, et al. Repression of hedgehog signal transduction in T-lineage cells increases TCR-induced activation and proliferation. Cell Cycle. 2008;7:904-908.
26. Rowbotham NJ, Hager-Theodorides AL, Furmanski AL, Crompton T. A novel role for Hedgehog in T-cell receptor signaling: implications for development and immunity. Cell Cycle. 2007;6:2138-2142.
27. de Boer J, Williams A, Skavdis G, et al. Transgenic mice with hematopoietic and lymphoid specific expression of Cre. Eur J Immunol. 2003;33:314-325.
28. Hager-Theodorides AL, Rowbotham NJ, Outram SV, Dessens JT, Crompton T. beta-Selection: Abundance of TCRbeta(-)/gammadelta(-) CD44(-)CD25(-) (DN4) cells in the foetal thymus. Eur J Immunol. 2007;37:487-500.
29. Gounari F, Chang R, Cowan J, et al. Loss of adenomatous polyposis coli gene function disrupts thymic development. Nat Immunol. 2005;6:800-809.
30. Bai CB, Auerbach W, Lee JS, Stephen D, Joyner AL. Gli2, but not Gli1, is required for initial Shh signaling and ectopic activation of the Shh pathway. Development. 2002;129:4753-4761.
31. Hoffman ES, Passoni L, Crompton T, et al. Productive T-cell receptor beta-chain gene rearrangement: coincident regulation of cell cycle and clonality during development in vivo. Genes Dev. 1996;10:948-962.
32. Neumann CJ. Hedgehogs as negative regulators of the cell cycle. Cell Cycle. 2005;4:1139-1140.
33. van den Brink GR. Hedgehog signaling in development and homeostasis of the gastrointestinal tract. Physiol Rev. 2007;87:1343-1375.
34. Locker M, Agathocleous M, Amato MA, Parain K, Harris WA, Perron M. Hedgehog signaling and the retina: insights into the mechanisms controlling the proliferative properties of neural precursors. Genes Dev. 2006;20:3036-3048.
35. Masai I, Yamaguchi M, Tonou-Fujimori N, Komori A, Okamoto H. The hedgehog-PKA pathway regulates two distinct steps of the differentiation of retinal ganglion cells: the cell-cycle exit of retinoblasts and their neuronal maturation. Development. 2005;132:1539-1553.
36. Agathocleous M, Locker M, Harris WA, Perron M. A general role of hedgehog in the regulation of proliferation. Cell Cycle. 2007;6:156-159.

37. Tenzen T, Allen BL, Cole F, Kang JS, Krauss RS, McMahon AP. The cell surface membrane proteins Cdo and Boc are components and targets of the Hedgehog signaling pathway and feedback network in mice. Dev Cell. 2006;10:647-656.
38. Yoon JW, Kita Y, Frank DJ, et al. Gene expression profiling leads to identification of GLI1-binding elements in target genes and a role for multiple downstream pathways in GLI1-induced cell transformation. J Biol Chem. 2002;277:5548-5555.
39. Shkumatava A, Neumann CJ. Shh directs cell-cycle exit by activating p57Kip2 in the zebrafish retina. EMBO Rep. 2005;6:563-569.
40. Varas A, Hager-Theodorides AL, Sacedon R, Vicente A, Zapata AG, Crompton T. The role of morphogens in T-cell development. Trends Immunol. 2003;24:197-206.
41. Cejalvo T, Sacedon R, Hernandez-Lopez C, et al. Bone morphogenetic protein-2/4 signalling pathway components are expressed in the human thymus and inhibit early T-cell development. Immunology. 2007;121:94-104.
42. Hager-Theodorides AL, Outram SV, Shah DK, et al. Bone morphogenetic protein 2/4 signaling regulates early thymocyte differentiation. J Immunol. 2002;169:5496-5504.
43. Graf D, Nethisinghe S, Palmer DB, Fisher AG, Merkenschlager M. The developmentally regulated expression of Twisted gastrulation reveals a role for bone morphogenetic proteins in the control of T cell development. J Exp Med. 2002;196:163-171.

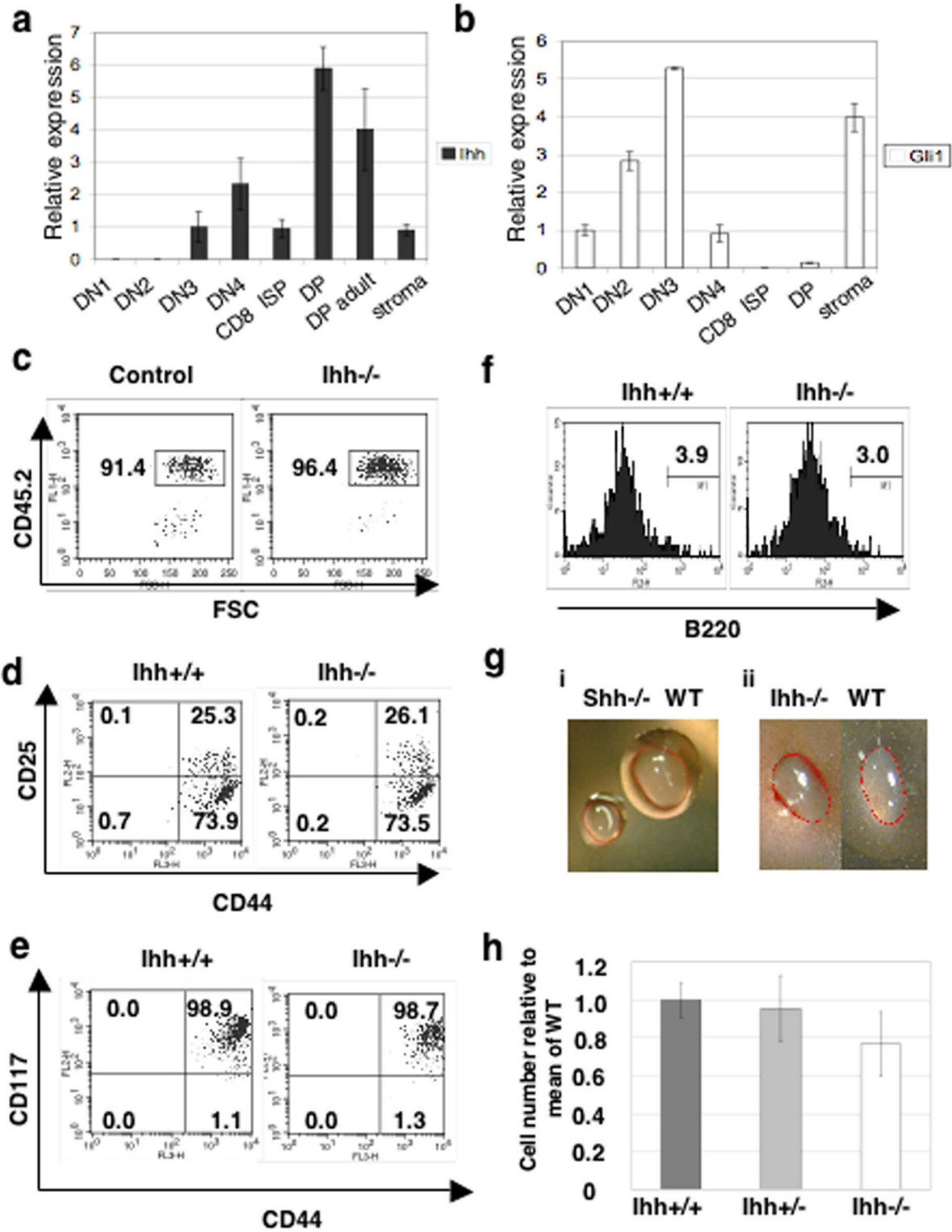

a
Relative expression
Ihh
DN1
DN2
DN3
DN4
CD8 ISP
DP
DP adult
stroma
b
Relative expression
Gli1
DN1
DN2
DN3
DN4
CD8 ISP
DP
stroma
c
Control
Ihh-/-
91.4
96.4
CD45.2
FSC
f
Ihh+/+
Ihh-/-
3.9
3.0
B220
d
Ihh+/+
Ihh-/-
0.1
25.3
0.7
73.9
0.2
26.1
0.2
73.5
CD25
CD44
g
i
Shh-/- WT
ii
Ihh-/- WT
e
Ihh+/+
Ihh-/-
0.0
98.9
0.0
1.1
0.0
98.7
0.0
1.3
CD117
CD44
h
Cell number relative to mean of WT
Ihh+/+
Ihh+/-
Ihh-/-

Figure 1

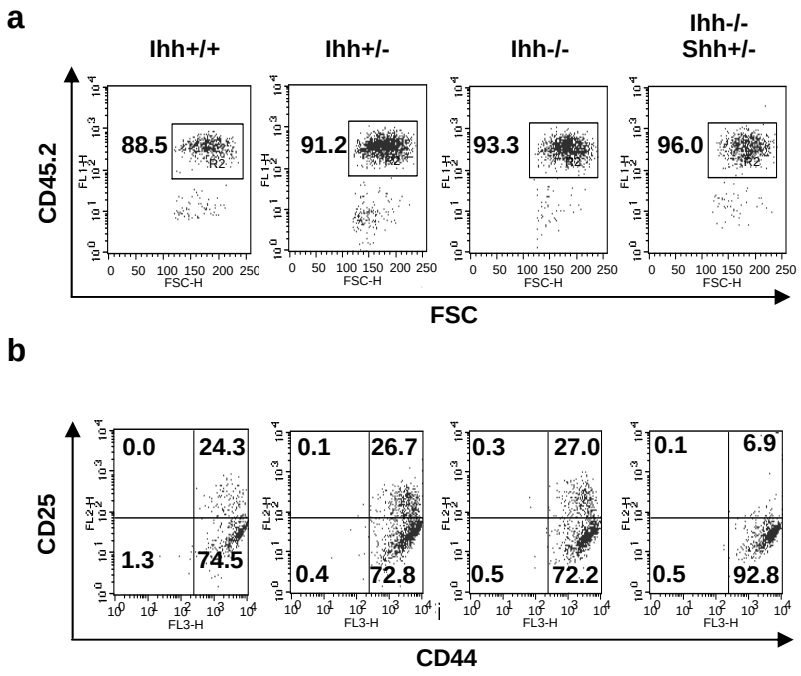

a
Ihh+/+
Ihh+/-
Ihh-/-
Ihh-/-
Shh+/-
CD45.2
88.5
91.2
93.3
96.0
R2
FL1-H
FSC-H
FSC
b
CD25
0.0
24.3
1.3
74.5
0.1
26.7
0.4
72.8
0.3
27.0
0.5
72.2
0.1
6.9
0.5
92.8
FL2-H
FL3-H
CD44

Figure 2

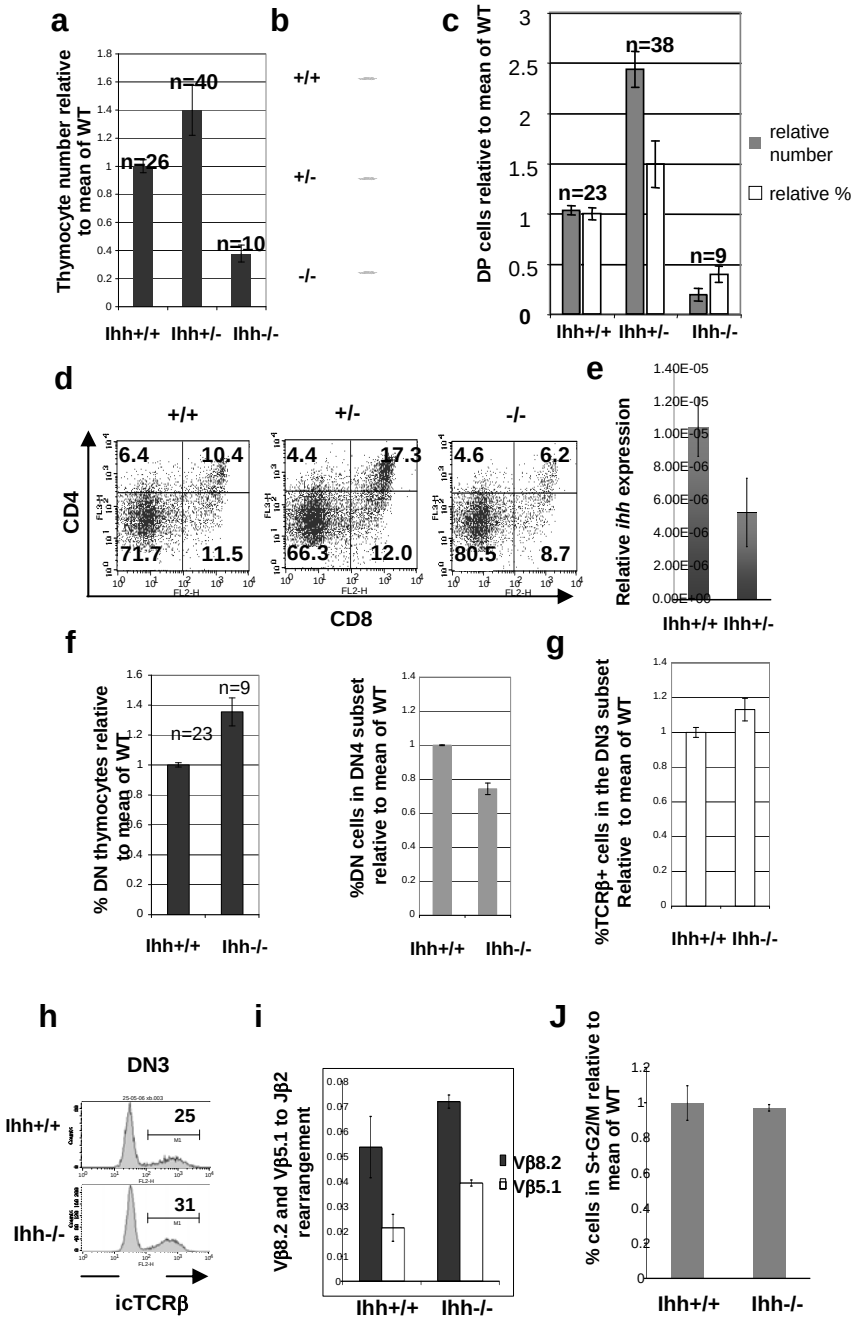

a
Thymocyte number relative to mean of WT
n=40
n=26
n=10
Ihh+/+ Ihh+/- Ihh-/-
b
+/+
+/-
-/-
c
DP cells relative to mean of WT
n=38
n=23
n=9
relative number
relative %
Ihh+/+ Ihh+/- Ihh-/-
d
+/+
+/-
-/-
CD4
CD8
6.4
10.4
71.7
11.5
4.4
17.3
66.3
12.0
4.6
6.2
80.5
8.7
e
Relative ihh expression
Ihh+/+ Ihh+/-
f
% DN thymocytes relative to mean of WT
n=9
n=23
Ihh+/+ Ihh-/-
%DN cells in DN4 subset relative to mean of WT
Ihh+/+ Ihh-/-
g
%TCRβ+ cells in the DN3 subset Relative to mean of WT
Ihh+/+ Ihh-/-
h
DN3
Ihh+/+
Ihh-/-
25
31
icTCRβ
i
Vβ8.2 and Vβ5.1 to Jβ2 rearrangement
Vβ8.2
Vβ5.1
Ihh+/+ Ihh-/-
J
% cells in S+G2/M relative to mean of WT
Ihh+/+ Ihh-/-

Figure 3

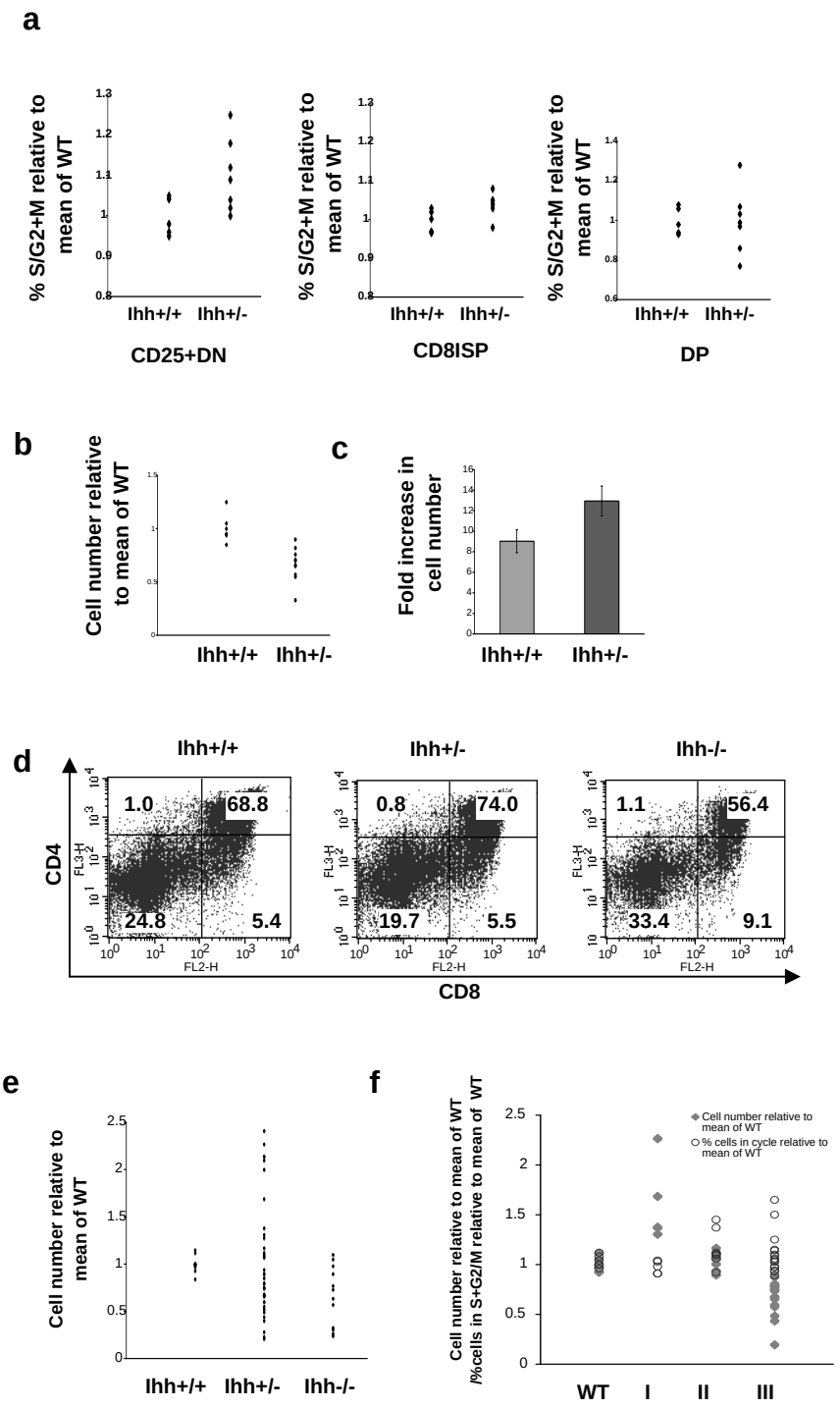
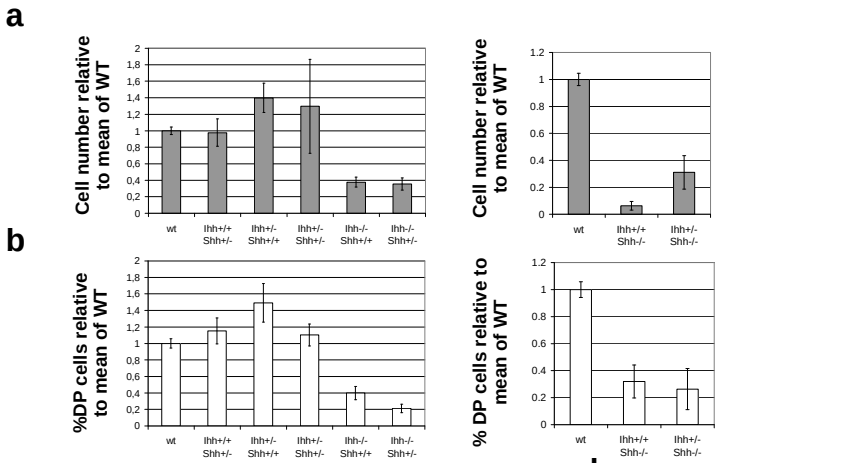
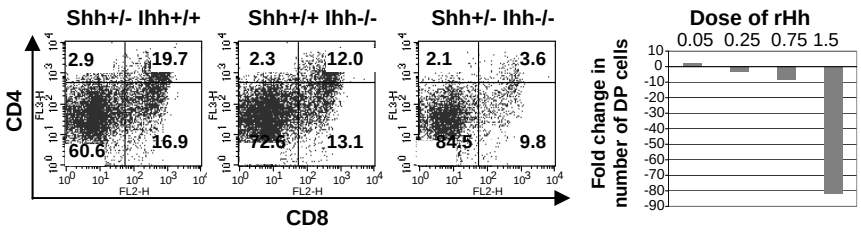
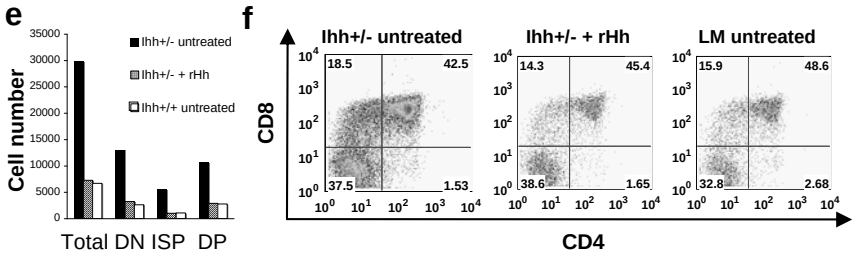

a
% S/G2+M relative to mean of WT
Ihh+/+
Ihh+/-
CD25+DN
CD8ISP
DP
b
Cell number relative to mean of WT
c
Fold increase in cell number
d
Ihh+/+
Ihh+/-
Ihh-/-
CD4
CD8
FL3-H
FL2-H
1.0
68.8
24.8
5.4
0.8
74.0
19.7
5.5
1.1
56.4
33.4
9.1
e
f
Cell number relative to mean of WT
% cells in cycle relative to mean of WT
Cell number relative to mean of WT /%cells in S+G2/M relative to mean of WT
WT
I
II
III

Figure 4


a
Cell number relative to mean of WT
wt
Ihh+/+ Shh+/-
Ihh+/- Shh+/+
Ihh+/- Shh+/-
Ihh-/- Shh+/+
Ihh-/- Shh+/-
Cell number relative to mean of WT
wt
Ihh+/+ Shh-/-
Ihh+/- Shh-/-
b
%DP cells relative to mean of WT
% DP cells relative to mean of WT


c
Shh+/- Ihh+/+
Shh+/+ Ihh-/-
Shh+/- Ihh-/-
2.9
19.7
60.6
16.9
2.3
12.0
72.6
13.1
2.1
3.6
84.5
9.8
CD4
CD8
FL2-H
FL3-H
d
Dose of rHh
0.05 0.25 0.75 1.5
Fold change in number of DP cells


e
Cell number
Ihh+/- untreated
Ihh+/- + rHh
Ihh+/+ untreated
Total DN ISP DP
f
Ihh+/- untreated
Ihh+/- + rHh
LM untreated
18.5
42.5
37.5
1.53
14.3
45.4
38.6
1.65
15.9
48.6
32.8
2.68
CD8
CD4

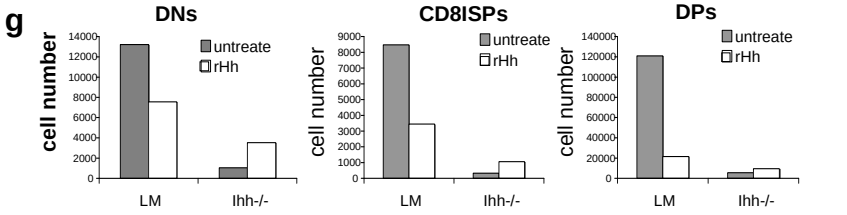

g
DNs
CD8ISPs
DPs
cell number
untreate
rHh
LM
Ihh-/-

Figure 5

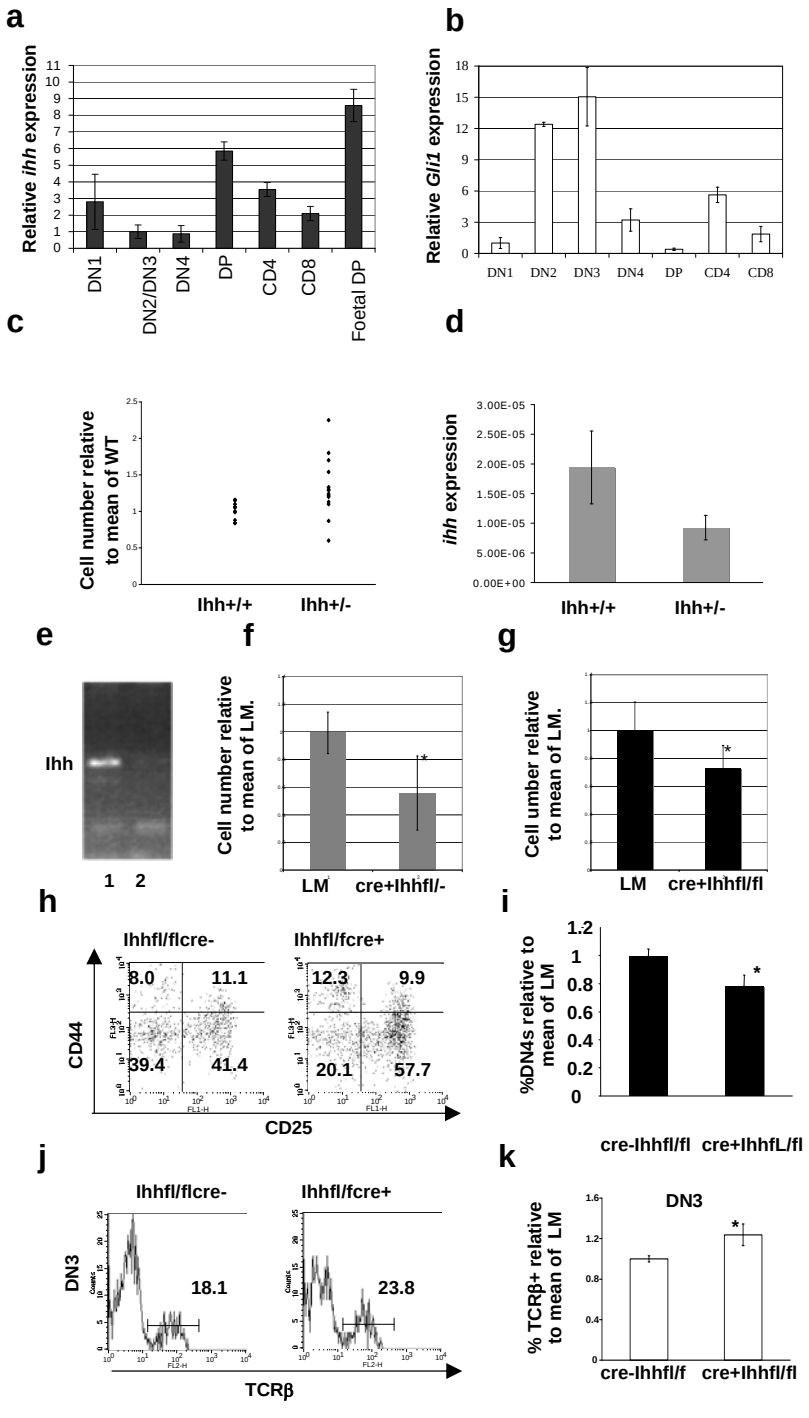

a
Relative ihh expression
DN1
DN2/DN3
DN4
DP
CD4
CD8
Foetal DP
b
Relative Gli1 expression
DN1
DN2
DN3
DN4
DP
CD4
CD8
c
Cell number relative to mean of WT
Ihh+/+
Ihh+/-
d
ihh expression
Ihh+/+
Ihh+/-
e
Ihh
1
2
f
Cell number relative to mean of LM.
LM
cre+Ihhfl/-
g
Cell umber relative to mean of LM.
LM
cre+Ihhfl/fl
h
Ihhfl/flcre-
Ihhfl/fcre+
8.0
11.1
39.4
41.4
12.3
9.9
20.1
57.7
CD44
CD25
i
%DN4s relative to mean of LM
cre-Ihhfl/fl
cre+IhhfL/fl
j
Ihhfl/flcre-
Ihhfl/fcre+
DN3
18.1
23.8
TCRβ
k
DN3
% TCRβ+ relative to mean of LM
cre-Ihhfl/f
cre+Ihhfl/fl

Figure 6

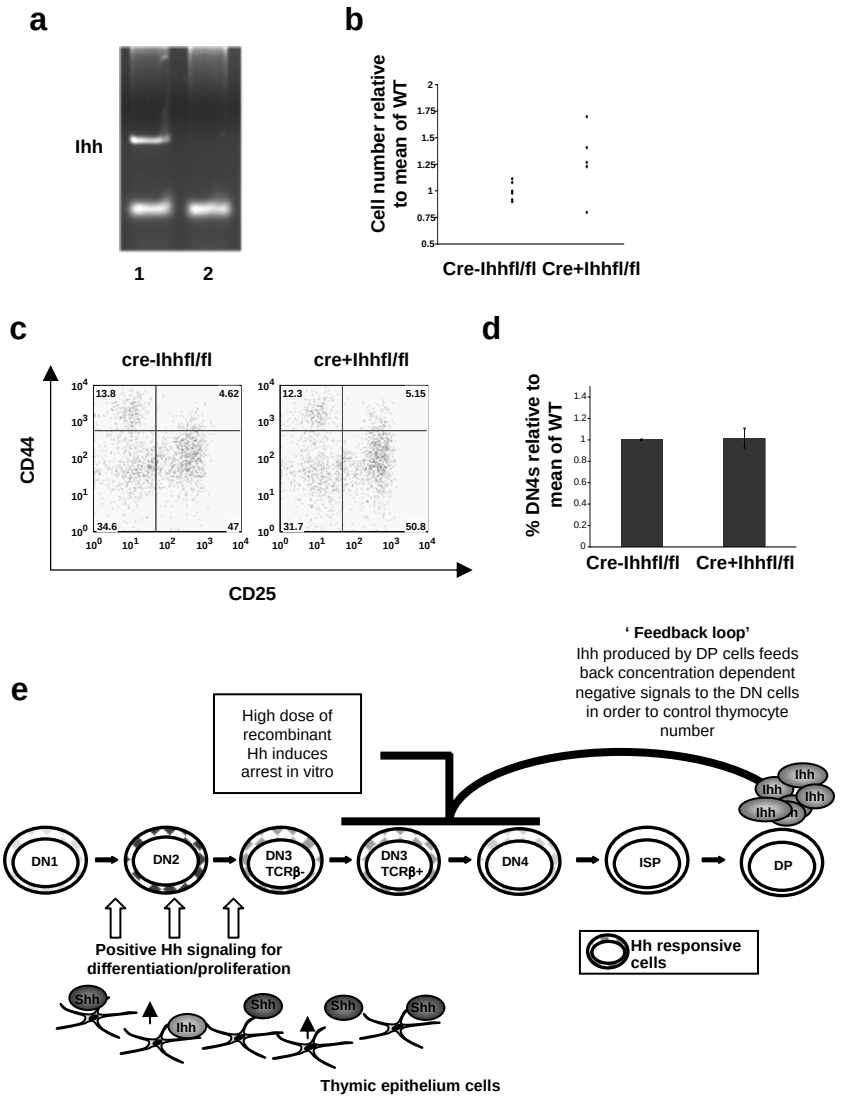

a
Ihh
1
2
b
Cell number relative to mean of WT
Cre-Ihhfl/fl
Cre+Ihhfl/fl
c
cre-Ihhfl/fl
cre+Ihhfl/fl
CD44
CD25
13.8
4.62
34.6
47
12.3
5.15
31.7
50.8
d
% DN4s relative to mean of WT
Cre-Ihhfl/fl
Cre+Ihhfl/fl
' Feedback loop'
Ihh produced by DP cells feeds back concentration dependent negative signals to the DN cells in order to control thymocyte number
e
High dose of recombinant Hh induces arrest in vitro
Ihh
DN1
DN2
DN3 TCRβ-
DN3 TCRβ+
DN4
ISP
DP
Positive Hh signaling for differentiation/proliferation
Hh responsive cells
Shh
Thymic epithelium cells

Figure 7