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Activation of the Hedgehog signaling pathway in T-lineage cells inhibits TCR repertoire selection in the thymus and peripheral T-cell activation

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TCR signal strength is involved in many cell fate decisions in the T-cell lineage. Here, we show that transcriptional events induced by Hedgehog (Hh) signaling reduced TCR signal strength in mice. Activation of Hh signaling in thymocytes in vivo by expression of a transgenic transcriptional-activator form of Gli2 (Gli2ΔN$_2$) changed the outcome of TCR ligation at many stages of thymocyte development, allowing self-reactive cells to escape clonal deletion; reducing transgenic TCR-mediated positive selection; reducing the ratio of CD4/CD8 single-positive (SP) cells; and reducing cell surface CD5 expression. In contrast, in the Shh$^{-/-}$ thymus the ratio of CD4/CD8 cells and both positive and negative selection of a transgenic TCR were increased, demonstrating that Shh does indeed influence TCR repertoire selection and the transition from double-positive (DP) to SP cell in a physiological situation. In peripheral T cells, Gli2ΔN$_2$ expression attenuated T-cell activation and proliferation, by a mechanism upstream of ERK phosphorylation. (Blood. 2007;109:3757-3766)

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Introduction

The Hedgehog (Hh) family of secreted intercellular signaling molecules specifies cell fate and patterning during embryonic development and is involved in homeostasis and renewal of adult tissues, including skin, gut, lung, blood, and thymus.[1-9]

The specialized environment of the thymus supports the maturation of lymphocyte precursors into functional T cells through stages defined by cell surface expression of developmentally regulated markers: CD4$^-$CD8$^-$ double-negative (DN) thymocytes differentiate to CD4$^+$CD8$^+$ double-positive (DP) cells that become mature CD4 or CD8 single-positive (SP) T cells. The DN population can be subdivided by expression of CD44 and CD25. The earliest CD44$^+$CD25$^-$ cells (DN1) acquire CD25 (DN2), then lose CD44 expression (DN3) and become CD44$^-$CD25$^-$DN (DN4) cells before differentiating to DP. The transition from DN to DP cell requires a functional pre-T-cell receptor (TCR) complex,[10] whereas maturation from DP to mature SP involves positive selection of the $\alpha\beta$TCR repertoire to ensure appropriate MHC restriction and negative selection of potentially self-reactive clones.[11,12] This developmental program is regulated by the stroma, and one way in which the epithelium signals to developing thymocytes is by production of Sonic Hh (Shh).[5]

The 3 mammalian Hh proteins (Shh, Indian Hh, and Desert Hh) share a common signaling pathway. Hh proteins bind their cell surface receptor Patched, releasing the signal transducer Smoothened (Smo) to transmit the Hh signal into the cell. At the end of this pathway are the transcription factors Gli1, Gli2, and Gli3.[1,2] Gli1 acts exclusively as an activator of transcription and, although not essential for mouse development or Hh signaling, is itself a Hh target gene, so measurement of its transcription can be used as a readout of Hh signaling in a given population of cells.[13] Gli2 and Gli3 undergo processing to function as positive or negative regulators of transcription, depending on the presence or absence of Hh signaling, respectively.[14] Both are essential for mouse development and have distinct but partially overlapping functions.[15,16] Gli2 functions primarily as a transcriptional activator downstream of Hh signaling, and is necessary to initiate the first transcriptional changes induced by the Hh signal.[17] In contrast, in vivo Gli3 functions primarily as a transcriptional repressor in the absence of the Hh signal.[18]

Analysis of Shh$^{-/-}$, Gli3$^{-/-}$, and Smo-deficient (conditional, T lineage) thymi has shown that Hh signaling is a positive regulator of the early stages of thymocyte development, controlling homeostasis of DN progenitors and differentiation from DN1 to DN2.[5,7,8] Both the analysis of the Shh$^{-/-}$[5] and Gli3$^{-/-}$[7] thymus and in vitro experiments using wild-type mouse thymus explants[4] have also suggested that Hh signaling is involved after TCR-β gene rearrangement at the transition from DN to DP cell. Surprisingly, however, conditional deletion of Smo from T-lineage cells failed to show any influence of loss of Hh signaling after the DN2 stage.[8] A role for Hh signaling has also been described in the activation of peripheral lymphocytes,[19,20] although no defect in T-cell activation was detected in Smo-deficient lymphocytes.[8]

Here, we investigated the function of Hh signaling at late stages of thymocyte development and in T-cell activation. By analysis of the Shh$^{-/-}$ thymus, we show that Shh influences TCR repertoire selection and the transition from DP to SP. We also show that T-cell–autonomous activation of the Hh pathway in developing thymocytes inhibited TCR repertoire selection, altering differentiation to SP and the CD4/CD8 SP ratio. In addition, we demonstrate that in mature T cells the transcriptional changes induced by Hh reduce the ability of the TCR to signal for activation and proliferation, by a mechanism upstream of ERK phosphorylation.

Materials and methods

Construction of lck-Gli2ΔN₂ transgenic

$Gli2\Delta N_2$ cDNA[14] was blunt-end cloned into the *Bam*HI site of the lck proximal promoter cassette.[21] The 8.9-kb transgene was isolated by *Not*I digestion and purified using QIAEXII Gel Extraction Kit (Qiagen, Hilden, Germany). CBA × C57BL/6 oocytes were injected.

Mice

lck-$Gli2\Delta N_2$ transgene-positive mice were backcrossed for more than 7 generations with C57BL/6 (B&K Universal, Grimston, England). Gli2ΔN₂ and Shh$^{+/-}$ mice were crossed with HY-TCR transgenic[22] and B6.21.6β-TCR transgenics.[23] Shh$^{+/-}$ mice[24] were on a C57BL/6 background.[5] Mice were maintained under United Kingdom Home Office regulations.

Genotyping

DNA was extracted using DNeasy tissue kit (Qiagen). Real-time polymerase chain reaction (PCR) for genomic $Gli2\Delta N_2$ was carried out on an iCycler (Bio-Rad Laboratories, Hercules, CA) using the iQSYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. Primers were Gli2F AGAACCTGAAGACACACCTGCG and Gli2R GAGGCATTGGAGAAGGCTTTG. To enable comparison between samples, $Gli2$ levels were normalized using levels of the $J1.1$ germ-line gene (primers were 5′J1.1GL CCTCATCCTATGGCACTG and 3′J1.1GL GCTTTGTCCGAA-GAGAGAC). Embryo sex was determined as described.[25]

Cell sorting

Thymocytes were sorted on a MoFlo (Cytomation, Fort Collins, CO). DN1-4 populations were sorted using antibodies against CD25^{FITC}, CD3/4/8^{PE}, and CD44^{Cychrome}. Staining with CD3^{FITC}, CD4^{PE}, and CD8^{Cychome} allowed sorting of DPs and SPs and CD8^{FITC}, CD4^{PE}, and B220^{Cychrome} allowed sorting of peripheral SPs and B cells. Cells collected fell within FSC/SSC live gate.

Quantitative RT-PCR for Gli2ΔN₂ transgene expression

RNA was extracted using Absolutely RNA Miniprep kit (Stratagene, La Jolla, CA) from fluorescence-activated cell sorter (FACS) cells. cDNA was synthesized using Superscript II (Invitrogen, Carlsbad, CA) and quantitative reverse-transcription (RT)–PCR carried out as described.[7] Primers were Gli2ΔN₂F AGAACCTGAAGACACACCTGCG and Gli2ΔN₂R GAGGCATTGGAGAAGGCTTTG.

Antibodies and flow cytometry

Cells were stained as described[7] using directly conjugated antibodies from BD PharMingen (San Diego, CA) and analyzed on a FACScan (BD Biosciences, Basel, Switzerland). Live cells were gated according to FSC/SSC profiles. Data are representative of more than 3 experiments.

CFSE (Sigma-Aldrich) labeling was carried out in PBS and 10 μM CFSE for 10 minutes at 25°C in the dark.

D^{b}Smcy^{PE} tetramer (ProImmune, Oxford, United Kingdom) staining was prior to other antibody staining in PBS for 15 minutes at 25°C in the dark.

In vitro T-cell activation

Splenic T cells were cultured at 5×10^6/mL in AIM-V (Life Technologies), 10^{-5} M 2ME (Sigma-Aldrich) at 37°C, 5% CO₂. Anti-CD3 and anti-CD28 (azide-free [NA/LE; BD PharMingen]) were at 0.01 μg/mL of each, unless otherwise stated.

Fetal thymus organ culture (FTOC)

FTOC was as described.[4] Modified recombinant human Shh, as described,[4] was a gift from Curis (Cambridge, MA).

Analysis of Erk1 and Erk2 phosphorylation

Splenic T cells at 5×10^6 in OptiMEM (Invitrogen) media were activated with 1 μg/mL anti-CD3ε and anti-CD28 antibodies and incubated on ice for 30 minutes. For stimulation, cells were transferred to 37°C for 5, 15, 30, 45, and 60 minutes in 5% CO₂. After a short spin, cells were lysed on ice for 40 minutes and analyzed by immunoblotting using anti-Erk1, anti-Erk2, anti–phospho-Erk, and anti–phospho-Erk2 (Cell Signaling, Beverly, MA) as described.[26]

For FACS analysis of intracellular phospho-Erk activation and staining were performed as described.[27]

Results

Expression of Gli1 in SP thymocytes

The Hh signaling pathway has previously been shown to be involved in regulation of thymocyte development at the transition from DN1 to DN2[5,7,8] and at the transition from DN to DP cell.[4,5,7] Genes encoding components of the signaling pathway, including Smo and Gli1, are expressed by DN thymocytes, and anti-Smo staining and RT-PCR analysis of transcription in sorted DN subsets have both shown that their expression is highest in the DN2 subset.[4,8] To assess activation status of the Hh signaling pathway at later stages of thymocyte development, we analyzed transcription of the Hh-target gene $Gli1$ in sorted thymocyte populations. We found that $Gli1$ transcription was down-regulated in the DN4 subset and DP population (Figure 1A). We did, however, detect an 11-fold increase in $Gli1$ transcription from the DP to CD4SP population, and a 4-fold increase from the DP to CD8SP population, indicating active Hh signaling at later stages of T-cell development, in SP cells (Figure 1A). The detection of $Gli1$ transcription in the CD4 and CD8 SP subsets is consistent with high cell surface expression of Smo on about 9% of CD4SP and 20% of CD8SP thymocytes.[4] Shh is expressed by epithelial cells in the medulla and corticomedullary junction,[4,6,8] and therefore expression of Gli1 and Smo in SP thymocytes is consistent with their
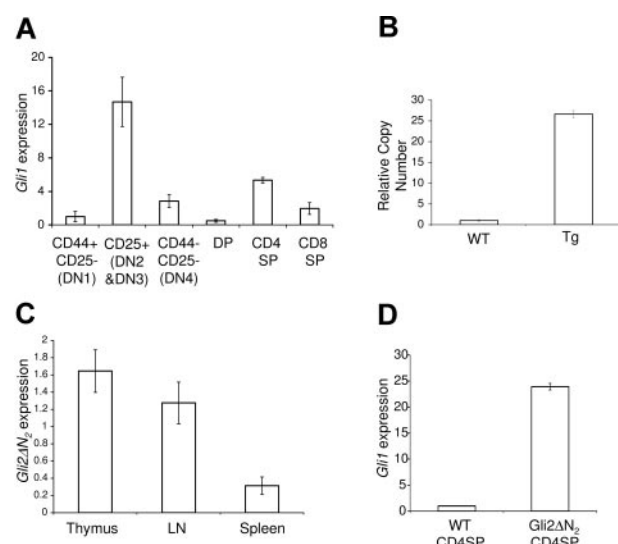

Figure 1. Expression of Gli1 and Gli2ΔN₂ in WT and Gli2ΔN₂ transgenic mice. (A) Hh activation status, shown by quantitative RT-PCR analysis of *gli1* mRNA in FACS-sorted populations of the adult murine thymus. (B) Quantitative PCR of genomic *Gli2ΔN₂,* showing high copy number of the Gli2ΔN₂ transgene. (C) Relative *Gli2ΔN₂* expression, by quantitative RT-PCR analysis, in thymus, lymph node, and spleen (D) and up-regulation of *Gli1* transcription in Gli2ΔN₂ transgenic CD4SP thymocytes. Error bars show standard deviation (SD). Data are representative of at least 3 experiments.

location in the thymus, as like the early DN populations,[28] they are located near the source of Shh.

Transgenic *Gli2* expression in thymocytes

Given that the Hh signaling pathway is active in SP thymocytes, we investigated the function of Hh signaling at later stages of thymocyte development. We made a transgenic mouse that expressed an N-terminally truncated form of Gli2 under the control of the lck promoter. This truncated form of Gli2 (Gli2ΔN$_2$) behaves as an activator of transcription independent of upstream effects on the pathway, thus constitutively activating Hh-dependent transcription in cells that express it,[14] and allowing study of the effect of pathway activation specifically in thymocytes. Use of this *lck* cassette restricts transgene expression to thymocytes without affecting the thymic stroma.[21] Two independent studies using the *lck* cassette to drive expression of a GFP reporter gene have shown transgenic expression first in the DN2 and DN3 subsets, and that full expression is achieved at DN4. Expression is maintained in DP, SP, and peripheral T-cell populations.[29,30]

Transgenic mice were produced and quantitative PCR of genomic DNA using primers that amplify both transgenic and genomic *Gli2* gene demonstrated high copy number (Figure 1B). Quantitative RT-PCR demonstrated *Gli2*ΔN$_2$ expression in transgenic thymocytes, splenocytes, and lymph node cells, with varying expression levels corresponding to the proportion of T-lineage cells

(Figure 1C). To confirm that the Gli2ΔN$_2$ protein was functional and that it activated transcription of Hh-target genes, we analyzed the transcription of *Gli1* in sorted CD4SP thymocytes from Gli2ΔN$_2$ transgenic and wild-type (WT) littermate thymi. We found an approximately 20-fold increase in *Gli1* transcription in transgenic thymocytes, confirming thymocyte-autonomous activation of the downstream transcriptional changes induced by Hh signaling (Figure 1D).

Hh signaling in the Gli2ΔN$_2$ transgenic influences the ratio of CD4/CD8 SP cells

We analyzed lymphocyte populations in Gli2ΔN$_2$ transgenic mice. Thymus, lymph nodes, and spleen were grossly normal, with no significant differences in lymphocyte numbers in thymus or spleen between Gli2ΔN$_2$ transgenic and WT littermates (Figure 2A). Analysis of CD4 and CD8 expression revealed a statistically significant reduction in the proportion of CD4SP cells and an increase in the proportion of CD8SP cells in the Gli2ΔN$_2$ transgenic thymus, relative to littermates. Typically, 6.8% of thymocytes were CD4SP and 2.6% were CD8SP in the Gli2ΔN$_2$ transgenic, compared with 9.9% and 1.7%, respectively, in WT littermates (Figure 2B), and the average ratio of CD4SP/CD8SP cells was reduced from 3.7 in WT to 2.3 in the Gli2ΔN$_2$ transgenic mice (Figure 2C). We also observed a significant increase in the percentage of DP cells in the Gli2ΔN$_2$ transgenic
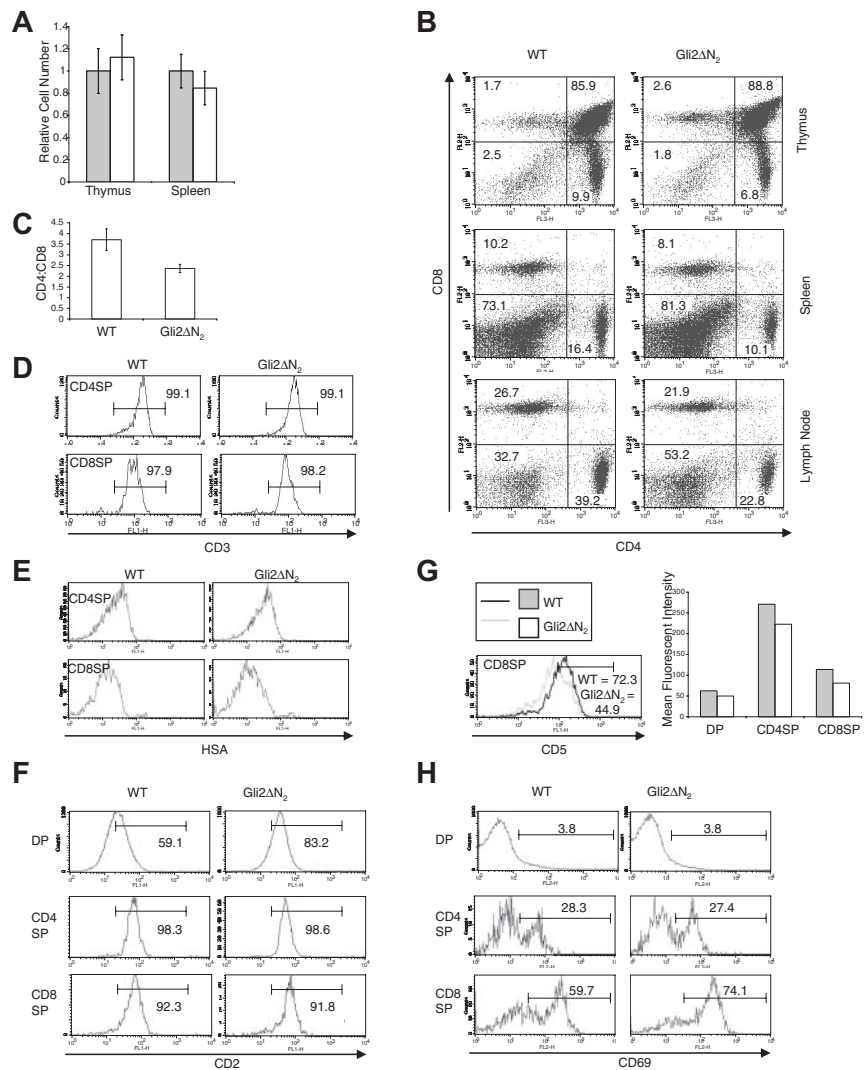

Figure 2. Phenotype of Gli2ΔN$_2$ transgenic mice. (A) Relative cell number (calculated relative to the mean of WT littermates) in the thymus and spleen of WT (gray bars, n = 6) and Gli2ΔN$_2$ transgenic ($\square$, n = 6). (B) CD4 and CD8 profiles in the Gli2ΔN$_2$ transgenic mouse. Thymocytes from Gli2ΔN$_2$ transgenic and WT littermates were analyzed for CD4 and CD8 expression in the thymus (top panels), spleen (middle panels), and lymph node (bottom panels). Differences between the percentage of populations between transgenic and littermate were statistically significant as given: (thymus) CD4SP, P = .005; DP, P = .014; (spleen) CD4, P = .002; CD8, P = .001; and (lymph node) CD4, P = .005; CD8, P = .041. (C) Bar chart to show change in CD4/CD8 ratio in thymus (WT [n = 6], Gli2ΔN$_2$ [n = 6], P = .032). (D) CD3 expression on CD4 (top histograms) and CD8 (bottom histograms) SP thymocytes. (E) HSA expression on CD4 (top) and CD8 (bottom) SP thymocytes. (F) CD2 expression on DP (top), CD4SP (middle), and CD8SP (bottom) cells in the Gli2ΔN$_2$ transgenic (right panels) and the WT (left panels). (G) CD5 expression on CD8SP cells in the Gli2ΔN$_2$ transgenic thymus (gray line) compared with the WT (black line). The bar chart shows mean MFI for WT (▤) and Gli2ΔN$_2$ transgenic ($\square$). (H) CD69 expression on DP (top), CD4 (middle), and CD8 (bottom) SP thymocytes. Numbers in dot plots or histograms indicate the percentage of cells falling in the quadrant, region, or marker. Error bars show standard error (SE). Data are representative of at least 3 experiments.

thymus, compared with littermates (Figure 2B). To determine if the change in CD4/CD8 ratio was genuinely the result of changes in the proportions of mature T cells rather than an increase in pre-DP immature CD8SP cells (ISPs), we analyzed CD3 and HSA expression. CD3 and HSA expression in the SP populations of Gli2ΔN_2 transgenic and WT littermate thymi were identical (Figure 2D-E), excluding an increase in the ISP population. In both spleen and lymph nodes, we found a reduction in the CD4/CD8 T-cell ratio and the overall percentage of CD4 and CD8 T cells (Figure 2B).

Strength of TCR signaling can influence CD4/CD8 lineage commitment in the thymus, with a strong TCR signal favoring differentiation to CD4SP.[31-34] We therefore analyzed expression of cell surface molecules associated with TCR signaling in thymocytes.[27] Expression of CD2[35] was significantly increased on the DP population in the Gli2ΔN_2 transgenic, from 59% in WT to 83% of DP cells in the Gli2ΔN_2 transgenic (Figure 2F), but was unaffected on SP populations. Expression of CD5, a molecule linked to attenuation of the TCR signal,[36] was lower on DP and SP populations in the Gli2ΔN_2 transgenic compared with WT littermates (Figure 2G). In the CD8SP population, 72% stained brightly in WT compared with 45% in the Gli2ΔN_2 transgenic, and mean fluorescence intensity (MFI) was reduced from 113.9 in the WT to 80.9 in the Gli2ΔN_2 transgenic (Figure 2G). Expression of CD69, a marker associated with repertoire selection and T-cell activation, was higher on CD8SP thymocytes in the Gli2ΔN_2 transgenic, with, in a typical experiment, 74% staining positive compared with 60% in the WT (Figure 2H).

Increased differentiation to CD4SP in the Shh$^{-/-}$ thymus

Given that activation of the Hh signaling pathway in developing thymocytes reduced the ratio of CD4/CD8 SP thymocytes, we asked if removal of the Shh signal would have the opposing effect. Shh$^{-/-}$ embryos die before birth[24] and have defects in DN thymocyte proliferation and differentiation, but in FTOC they accumulate a normal proportion of DP cells.[5] We therefore cultured E14.5 Shh$^{-/-}$ and littermate thymi and analyzed the production of CD4SP cells. After 3 and 6 days in culture, the Shh$^{-/-}$ thymi contained a significantly greater proportion of CD4SP cells than those of littermates, indicating that Shh negatively regulates the transition from DP to CD4SP cell (Figure 3A-C). In E14.5+6-day FTOC, only 30% of SP cells express high cell surface CD3.[5] Therefore to confirm that the increase in the CD4SP population was due to the accumulation of mature T cells, we also analyzed differentiation to SP cells in E17.5 Shh$^{-/-}$ and littermate FTOC after 7 days. On E17.5, the thymus contained approximately 70% DP cells and there was no significant difference in the proportion of DP cells between Shh$^{-/-}$ and littermate thymi. Mature SP cells had not arisen, facilitating investigation of the transition from DP to SP. In a typical experiment, the ratio of CD4/CD8 SP cells was increased from 2.6 in the Shh$^{+/+}$ to 3.4 in the Shh$^{-/-}$ FTOC. The proportion of CD4SP cells was significantly increased from 13% in Shh$^{+/+}$ FTOC to 21.2% in Shh$^{-/-}$ FTOC (Figure 3D). There was no difference in the proportion of CD3^{high} cells in each SP population between Shh$^{-/-}$ and Shh$^{+/+}$ littermate cultures, indicating that the increase in CD4SP cells was genuinely due to the accumulation of mature cells (Figure 3D). Of interest, CD69 expression was increased in the CD4SP Shh$^{-/-}$ population (Figure 3D). Therefore, under all culture conditions the Shh$^{-/-}$ thymus favored the production of CD4SP over CD8SP cells (Figure 3E).

When we compared the ratio of SP/DP in the Shh$^{-/-}$ thymus to that of littermates, we found an increased ratio under all culture conditions. Thus, despite the smaller size of the Shh$^{-/-}$ thymus,[5] the DP population gave rise to relatively more SP cells (Figure 3F).

Shh treatment reduced the CD4/CD8 ratio and CD5 expression in thymus explants

Given that the production of mature CD4SP cells was increased in the Shh$^{-/-}$ thymus, we asked if treatment of WT FTOC with
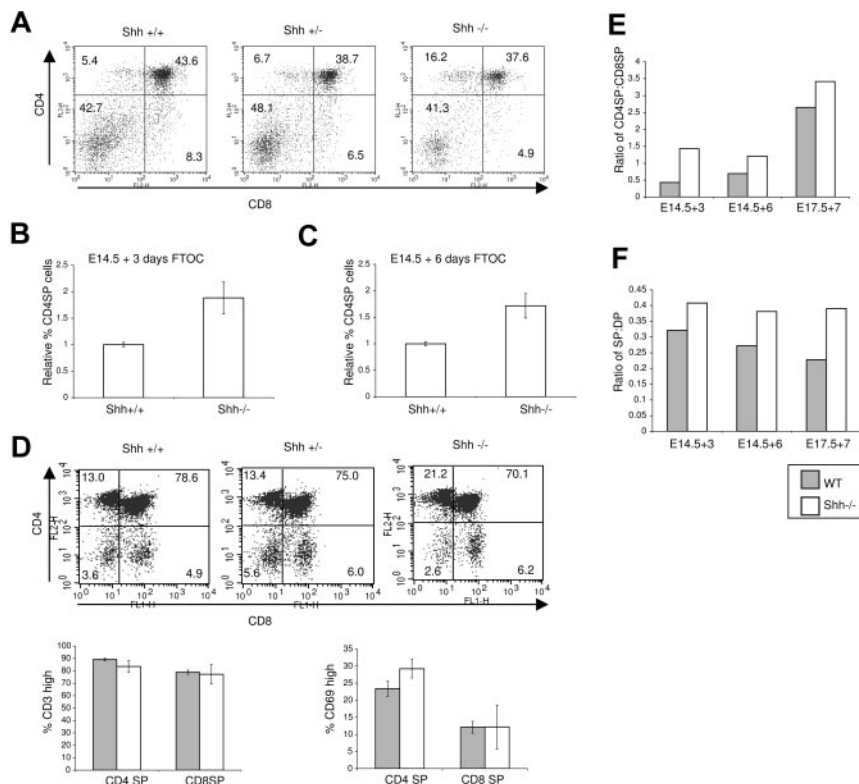

Figure 3. Increased differentiation to CD4SP in the Shh$^{-/-}$ thymus. (A) CD4 and CD8 staining of E14.5 Shh$^{-/-}$, Shh$^{+/-}$, and Shh$^{+/+}$ FTOC littermates, cultured for 6 days. Mean thymus sizes were 2.3×10^4 for Shh$^{-/-}$, 1.6×10^5 for Shh$^{+/-}$, and 1.8×10^5 for Shh$^{+/+}$. (B-C) Bar charts to show the relative proportion of CD4SP thymocytes in E14.5 Shh$^{-/-}$ and littermate FTOC after 3 (B) and 6 (C) days in culture. For each thymus, the proportion of CD4SP cells was calculated relative to the mean proportion in WT littermates. The difference in the relative proportion of CD4SP cells between Shh$^{-/-}$ and Shh$^{+/+}$ thymi was statistically significant: $P = .006$ for 3-day cultures (WT [n = 7], Shh$^{-/-}$ [n = 5]), $P = .022$ for 6-day cultures (WT [n = 7], Shh$^{-/-}$ [n = 6]). (D) CD4 and CD8 staining of E17.5 Shh$^{-/-}$, Shh$^{+/-}$, and Shh$^{+/+}$ FTOC littermates, cultured for 7 days. The average percentage of CD4SPs in the Shh$^{-/-}$ was increased 1.4 times compared with littermates ($P = .046$). The ratio of CD4/CD8 SP cells was 2.6 in Shh$^{+/+}$ FTOC and 3.4 in Shh$^{-/-}$ FTOC. Bar charts to show CD3 and CD69 expression on CD4 and CD8 SP thymocytes in the Shh$^{-/-}$ ($\square$, n = 3) and littermates ($\blacksquare$, n = 14). Mean thymus sizes were 9×10^4 for Shh$^{-/-}$, 1.3×10^5 for Shh$^{+/-}$, and 1.9×10^5 for Shh$^{+/+}$. (E) Bar chart to show mean CD4/CD8 ratio for the different culture conditions. (F) Bar chart to show mean SP/DP ratio for the different culture conditions. Error bars show SE. Data are representative of at least 3 experiments.

recombinant Shh (r-Shh) could reduce differentiation to CD4SP. We treated E17.5 FTOC with r-Shh for 7 days. Typically, the percentage of DP cells in r-Shh–treated cultures increased to 78.5% compared with 70.7% in the control, whereas the proportion of CD4SP cells was decreased from 15% in control to 9.3% in treated cultures. When we compared the ratio of mature (CD3^{high}) CD4/CD8 SP cells (Figure 4A), we found a dose-dependent reduction with maximum inhibition at 0.05 μg/mL r-Shh. CD5 expression was reduced in r-Shh–treated FTOC relative to controls in DP and SP populations (Figure 4B), consistent with the duller expression observed in the Gli2ΔN$_2$ transgenic thymus (Figure 2G).

When E17.5 FTOC were cultured for 10 days, Shh treatment again reduced differentiation from DP to CD4SP (Figure 4C). Gating on CD3^{high} cells revealed a relative increase in the proportion of CD8SP cells from 14.9% in the control to 27.1% in Shh-treated cultures, concomitant with the decrease in the CD4SP population. Overall, the ratio of mature (CD3^{high}) CD4/CD8 SP cells was reduced from 4.7 in the control to 2.1 in the Shh-treated cultures.

Taken together, these data show that Shh signaling is involved in regulating differentiation from DP to SP and CD4/CD8 ratio. Absence of Shh increased production of CD4SP cells, but Hh pathway activation, both by thymocyte autonomous Gli2ΔN$_2$ expression in vivo and by treatment with r-Shh in vitro, decreased cell surface CD5 expression and the production of CD4SP cells.

Negative selection is compromised in the Gli2ΔN$_2$HY-TCR transgenic male thymus

The pattern of expression of CD2, CD5, and CD69 in the Gli2ΔN$_2$ thymus together with the opposing effects of removal or activation of the Shh signal on differentiation from DP to CD4SP cell suggested that Hh signaling influences the later stages of thymocyte development by a TCR-dependent mechanism, and that activation of the Hh signaling pathway in thymocytes might lower the strength of the TCR signal. Therefore, to assess the effect of Hh signaling on repertoire selection we crossed the Gli2ΔN$_2$ transgenic with transgenic mice expressing the HY-TCR.[22] The HY-TCR recognizes a male-specific peptide from the *Smcy* gene presented by H-2D^b,[37] allowing assessment of negative selection in male mice.[22] As expected, male HY-TCR mice showed efficient clonal deletion of CD8SP and DP cells (Figure 5A). However, in

the Gli2ΔN$_2$HY-TCR thymus we found an increase in the percentage of DP cells and the presence of a small population of CD8SPs that had escaped deletion (Figure 5A). This population made up 2.5% of thymocytes and was absent in HY-TCR littermates. It expressed high levels of CD8 and CD69, and stained bright with anti-Vβ8 and D^b*Smcy* tetramer, confirming that it expressed the transgenic male-reactive TCR and had genuinely escaped negative selection (Figure 5A). We found that a small proportion (~ 3%) of peripheral lymph node cells expressed high levels of CD8 and stained brightly with D^b*Smcy* tetramer in the Gli2ΔN$_2$HY-TCR mice, which were absent in male HY-TCR littermates (Figure 5B)

In the male HY-TCR transgenic model, negative selection in the thymus can commence at an earlier developmental stage than would occur physiologically because of early expression of the αβTCR in DN cells.[38] To confirm that inhibition of clonal deletion was not restricted to early TCRα expression, we analyzed the effect of the Gli2ΔN$_2$ transgene on negative selection in male TCRβ chain–only transgenic mice, B6.2.16β.[23] The B6.2.16β mice express the same TCRβ chain as the HY-TCR transgenic, but the TCRα chain is variable and male-reactive D^b*Smcy*-binding T cells are deleted in the male animals. The proportion of CD8SP cells (all of which expressed the transgenic β chain) that stained brightly with D^b*Smcy* tetramer in the lymph node was more than doubled in the presence of the Gli2ΔN$_2$ transgene (Figure 5C). Thus, clonal deletion of self-reactive thymocytes was also compromised in Gli2ΔN$_2$B6.2.16β male mice.

Hh signaling influences positive selection in HY-TCR transgenic female mice

In the female Gli2ΔN$_2$HY-TCR thymus, we found an increased proportion of CD8SPs compared with the control HY-TCR female, together with a decrease in the CD4SP population (Figure 5D), as observed in the non-TCR transgenic Gli2ΔN$_2$ thymus. On first consideration, it therefore seemed that the Gli2ΔN$_2$ transgene might actually be enhancing positive selection of the transgenic αβTCR to the CD8 lineage. However, when we gated on D^b*Smcy*-[bright] cells (Figure 5D), we consistently found a reduction in the proportion of CD8SP cells and increase in DP cells in the Gli2ΔN$_2$HY-TCR thymus compared with littermates, indicative of
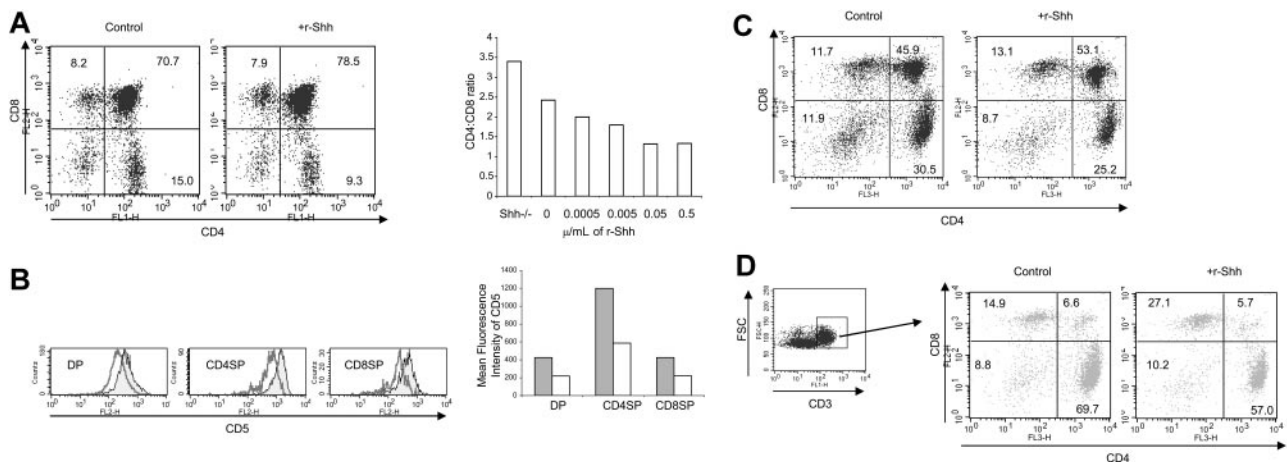

Figure 4. Shh treatment reduced CD5 expression and the CD4/CD8 ratio in thymus explants. (A) CD4 and CD8 profiles of 7-day C57BL/6 E17.5 FTOC control and treated with 0.05 μg/mL r-Shh. The bar chart shows the mean ratio of mature (CD3^{high}) CD4/CD8 SP in 7-day FTOC for Shh$^{-/-}$ and C57BL/6 E17.5 cultured with the stated concentration of recombinant Shh (r-Shh). For all treatments, n = 4. Mean cell recovery was as follows: control, 1.87 × 10^5; 0.5 μg/mL r-Shh, 1.66 × 10^5; 0.05 μg/mL r-Shh, 2.07 × 10^5; 0.005 μg/mL r-Shh, 2.1 × 10^5; and 0.0005 μg/mL r-Shh, 1.54 × 10^5. (B) CD5 expression on r-Shh–treated FTOC. Overlaid histograms of CD5 expression gated on the subsets from the FACS plots above for control cultures (gray filled) and r-Shh–treated cultures (gray line only, no fill). MFIs were (control/+ r-Shh) as follows: DP, 424.5/219.6; CD4SP, 1197.7/586.6; and CD8SP, 425.3/220.8. The bar chart shows MFI for the control (▨) and Shh-treated (▢) cultures. (C) CD4 and CD8 profiles of 10-day C57BL/6 E17.5 FTOC control and treated with 0.5 μg/mL r-Shh. Mean cell recovery was as follows: control, 1.34 × 10^5; 0.5 μg/mL r-Shh, 1.44 × 10^5. (D) Profiles of CD4 and CD8, gated on CD3^{high} of 10-day C57BL/6 FTOC control and treated with 0.5 μg/mL r-Shh.
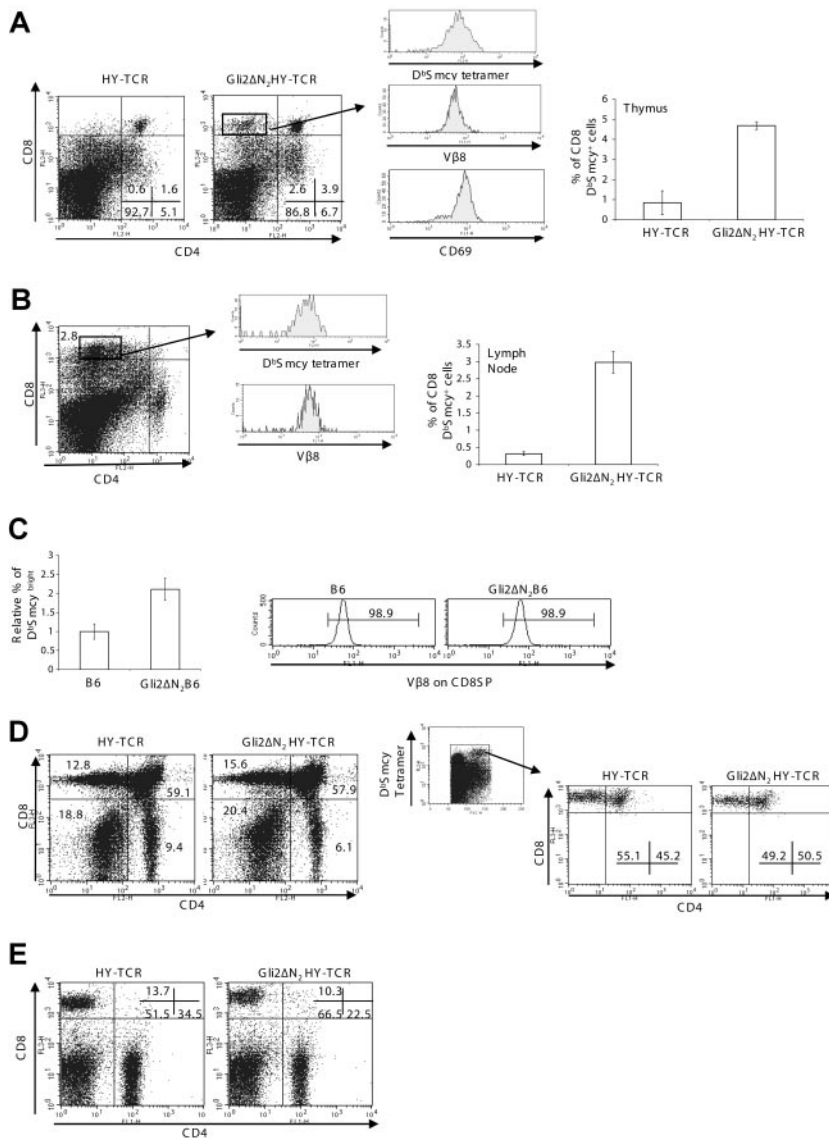
Figure 5. Gli2ΔN₂ expression influences TCR repertoire selection. (A) Thymus phenotype of male mice with the transgenic HY-TCR. CD4 and CD8 expression in HY-TCR–positive mice with (Gli2ΔN₂HY-TCR) and HY-TCR littermates without the Gli2ΔN₂ transgene. Mean thymus sizes were 2.4×10^7 for HY-TCR and 2.3×10^7 for Gli2ΔN₂HY-TCR. The histograms show $D^b Smcy$ tetramer, Vβ8, and CD69 staining on CD8SP cells from the Gli2ΔN₂HY-TCR. The bar chart shows the percentage of CD8 $D^b Smcy$-positive cells in the HY-TCR (n = 12) and Gli2ΔN₂HY-TCR (n = 8) thymus ($P = < .001$). (B) Peripheral phenotype of male mice with the HY transgenic TCR. CD4 and CD8 profile of a Gli2ΔN₂HY-TCR lymph node. The bar chart shows the percentage of CD8 $D^b Smcy$-positive cells in HY-TCR (n = 12) and Gli2ΔN₂HY-TCR (n = 8) lymph node ($P = < .001$). The histograms show $D^b Smcy$ and Vβ8 expression on CD8 T cells from the Gli2ΔN₂HY-TCR. (C) Lymph node of male mice with the B6.2.16β transgenic TCR. Bar chart to show the relative number of CD8⁺ $D^b Smcy^{bright}$ cells in B6.2.16β-positive mice (B6) and B6.2.16βGli2ΔN₂-positive mice (Gli2ΔN₂B6) ($P = .021$, n = 4, n = 5, respectively). The histograms show Vβ8 expression on CD8 T cells. (D) Thymus phenotype of HY-TCR female mice. CD4 and CD8 profiles of HY-TCR and Gli2ΔN₂HY-TCR mice (left dot plots). Mean thymus sizes were 8.7×10^7 for HY-TCR and 8.4×10^7 for Gli2ΔN₂HY-TCR. CD4 and CD8 profiles gated on $D^b Smcy$-positive cells in the HY-TCR and Gli2ΔN₂HY-TCR thymus (right dot plots). The percentages of cells falling in the $D^b Smcy$-positive gate were typically 9.6% in the HY-TCR and 9.4% in the Gli2ΔN₂HY-TCR. (E) Peripheral phenotype of HY-TCR female mice. CD4 and CD8 profiles in HY-TCR and Gli2ΔN₂HY-TCR mice. Numbers in dot plots or histograms indicate the percentage of cells falling in the quadrant, region, or marker. Error bars show SE. Data are representative of at least 3 experiments.

reduced positive selection of the transgenic αβTCR. In a representative experiment the percentage of $D^b Smcy^{bright}$ cells that was CD8SP was reduced from 55.1% in the control HY-TCR thymus to 49.2% in the Gli2ΔN₂HY-TCR thymus, whereas the total percentage of $D^b Smcy^{bright}$ thymocytes was unaffected (Figure 5D).

In lymph nodes, the Gli2ΔN₂ transgene caused a reduction in CD4 and CD8 peripheral T cells. The CD8 population was reduced from 14% to 10% and the CD4SP population from 35% to 23% (Figure 5E), suggesting that Gli2ΔN₂ expression also affected peripheral T-cell expansion and homeostasis.

Altered TCR repertoire selection in Shh mutant thymus

To ask if Shh affects TCR repertoire selection in a physiological situation, we introduced the transgenic HY-TCR onto the Shh⁺/⁻ background and compared positive selection in young adult female Shh⁺/⁻ HY-TCR and their littermate thymi. We found increased positive selection of the transgenic TCR to the CD8 lineage in the Shh⁺/⁻ HY-TCR thymus (Figure 6A) with an overall increase in the proportion of CD8SP thymocytes from 11% to 16%. When we gated on $D^b Smcy^{bright}$ cells (Figure 6A) the proportion of CD8SP cells was consistently increased, typically from 39% in the Shh⁺/⁺ littermate to 49% in the Shh⁺/⁻ thymus, whereas the proportion of

DP cells was decreased from 60% in the Shh⁺/⁺ to 49% in the Shh⁺/⁻ thymus.

To analyze positive selection in the complete absence of Shh, we cultured female E17.5 Shh⁺/⁺ HY-TCR and Shh⁻/⁻ HY-TCR littermate FTOC for 10 days. The proportion of HY-TCR⁺ CD8SP cells was increased approximately 3-fold ($P = .027$), from 8% in the Shh⁺/⁺ HY-TCR to 23% in the Shh⁺/⁻ HY-TCR thymus (Figure 6B). Thus, in contrast to the Gli2ΔN₂HY-TCR thymus, we found that in both the embryo and the adult, mutation of Shh increased positive selection of the HY-TCR.

In the adult male HY-TCR thymus, clonal deletion is efficient and complete (Figure 5A and Kisielow et al[22]), and so we could not test if reduction in Shh copy number increased deletion. In the embryo, clonal deletion starts at E18 and a significant proportion of DP and CD8SP cells persists at birth and in the neonate.[39,40] We therefore analyzed negative selection in the fetal and neonatal thymus.

In 12-day-old male Shh⁺/⁺ HY-TCR thymi, we detected a small population of HY-TCR⁺ CD8SP (~ 5%) and DP (~ 4%) cells, which were significantly reduced ($P = .019$) to approximately 3.5% and 2%, respectively, in Shh⁺/⁻ HY-TCR littermates, indicating more efficient clonal deletion in the Shh⁺/⁻

Figure 6. Altered TCR repertoire selection in Shh mutant thymus. (A) Thymus phenotype of 4-week-old Shh$^{+/-}$ HY-TCR female mice. CD4 and CD8 profiles of HY-TCR and Shh$^{+/-}$ HY-TCR littermate mice (left dot plots). Mean thymus sizes were 3.7×10^7 for HY-TCR and 4.8×10^7 for Shh$^{+/-}$ HY-TCR. CD4 and CD8 profiles gated on D^{b}Smcy-positive cells in the HY-TCR and Shh$^{+/-}$ HY-TCR thymus (right dot plots). (B) Thymus phenotype of E17.5 Shh$^{-/-}$ HY-TCR female FTOC cultured for 10 days. CD4 and CD8 profiles of HY-TCR and Shh$^{-/-}$ HY-TCR littermate FTOC. Bar chart shows the relative percentage of transgenic (Tg) TCR$^+$ CD8^{bright} SP cells for HY-TCR (n = 3) and Shh$^{-/-}$ HY-TCR (n = 3); $P = .027$. The histograms show Vβ8 and T3.70 (transgenic TCRα chain) staining on CD8SP cells. (C) Thymus phenotype of 12-day-old Shh$^{+/-}$ HY-TCR male. CD4 and CD8 profiles of HY-TCR and Shh$^{+/-}$ HY-TCR littermates. Mean thymus sizes were 2.4×10^7 for HY-TCR and 2.1×10^7 for Shh$^{+/-}$ HY-TCR. Bar charts show the relative percentage of transgenic (Tg) TCR$^+$ CD8^{bright} SP or DP cells for HY-TCR (n = 3) and Shh$^{+/-}$ HY-TCR (n = 3); $P = .019$. The histograms show Vβ8 and T3.70 expression on CD8SP cells. (D) Thymus phenotype of E19 Shh$^{-/-}$ HY-TCR male. CD4 and CD8 profiles of HY-TCR, Shh$^{+/-}$ HY-TCR, and Shh$^{-/-}$ HY-TCR littermates. Mean thymus sizes were 2.5×10^5 for HY-TCR, 2.4×10^5 for Shh$^{+/-}$ HY-TCR, and 1.1×10^4 for Shh$^{-/-}$ HY-TCR. Bar charts show the relative percentage of Tg TCR$^+$ CD8^{bright} SP or DP cells for HY-TCR (n = 4), Shh$^{+/-}$ HY-TCR (n = 4, $P = .034$), and Shh$^{-/-}$ HY-TCR (n = 3, $P = .018$). The histograms show D^{b}Smcy and T3.70 staining on CD8SP cells. Numbers in dot plots indicate the percentage of cells falling in the quadrant. Error bars show SE. Data are representative of at least 3 experiments.
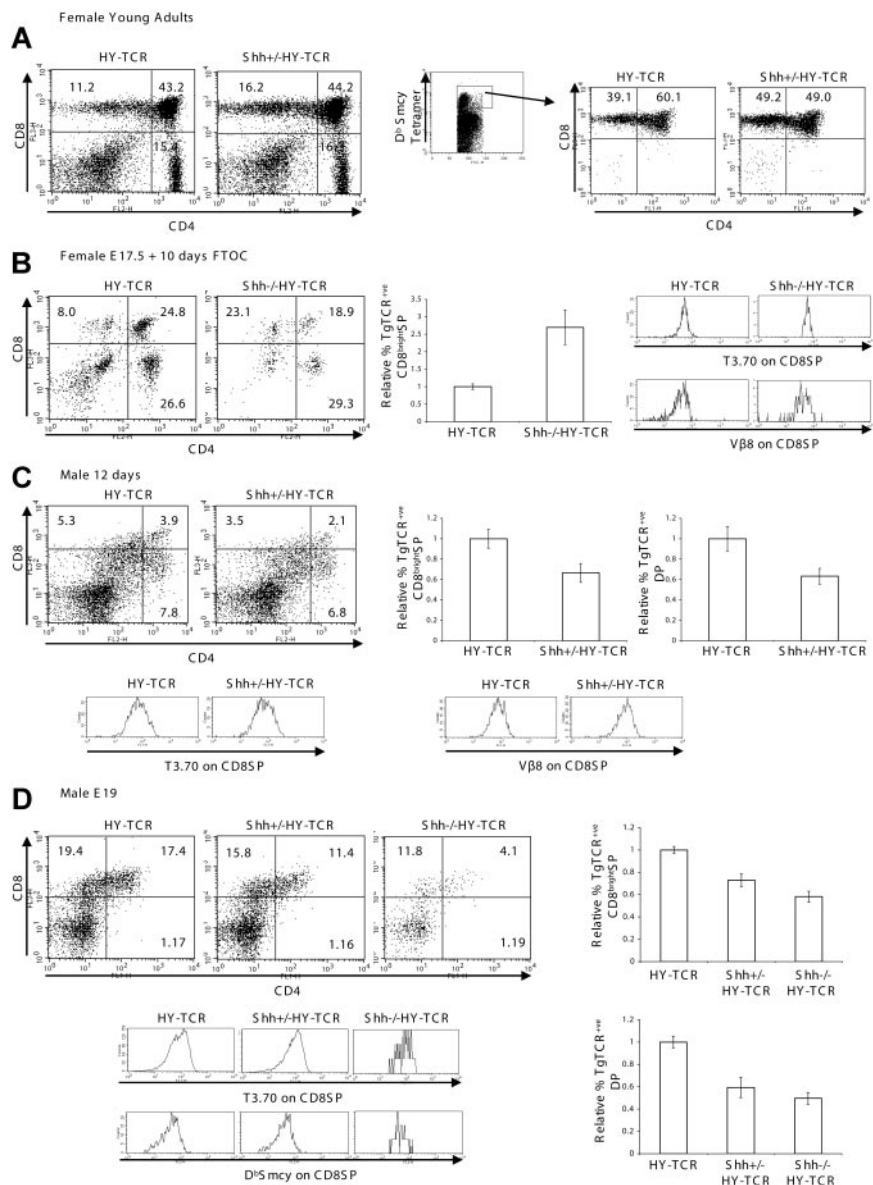
thymus (Figure 6C). On E19, the proportion of HY-TCR$^+$ CD8SP and DP cells was significantly reduced ($P = .018$) from 19% and 17%, respectively, in the Shh$^{+/+}$ thymus to only 12% and 4% in the Shh$^{-/-}$ littermate thymus (Figure 6D). The Shh$^{+/-}$ thymus gave an intermediate phenotype ($P = .034$). Therefore, we again found that removal of Shh increased the efficiency of TCR repertoire selection, in this case increasing negative selection of the self-reactive transgenic TCR. These data support the hypothesis that under physiological conditions, Shh modulates TCR signal strength in developing thymocytes.

Attenuated T-cell activation in peripheral Gli2ΔN$_2$ transgenic T cells

Given that activation or reduction of Hh signaling altered the outcome of TCR ligation in thymocytes, we investigated the effect of Gli2ΔN$_2$ expression on peripheral T-cell activation. We confirmed *Gli2ΔN$_2$* transgene expression and up-regulation of *Gli1* transcription by quantitative RT-PCR in sorted CD4 and CD8 T-cell populations (Figure 7A). As expected from previous expression studies,[29,30] the transgene was highly expressed in CD4 and CD8 T cells, and not detected in B cells. Transcription of *Gli1* was up-regulated in both T-cell populations, confirming

functional activity of the transgene. To assay T-cell activation, we stimulated splenocytes with anti-CD3 and anti-CD28 antibodies. After 24 hours, induction of cell surface expression of the early activation marker CD69 was inhibited in both CD4 and CD8 T cells from Gli2ΔN$_2$ transgenic spleens. In a typical experiment, only 18% of Gli2ΔN$_2$ transgenic CD4 T cells expressed CD69, compared with more than 60% of WT CD4 T cells (Figure 7B). Expression of the later activation marker CD25 was also decreased in Gli2ΔN$_2$ transgenic cultures on both CD4 and CD8 T cells, from 47% and 72% in WT CD4 and CD8 T populations, respectively, to 17% and 9% in their Gli2ΔN$_2$ transgenic counterparts (Figure 7B). These data show that activation of Hh signaling in T cells attenuated T-cell activation.

Proliferation of activated T cells is compromised by the Gli2ΔN$_2$ transgene

To assess proliferation, splenocytes were labeled with CFSE and stimulated with anti-CD3 and anti-CD28. The Gli2ΔN$_2$ transgenic T cells were unable to proliferate as well as their WT counterparts (Figure 7C). After 96 hours, only 20% of Gli2ΔN$_2$ transgenic CD4 T cells had undergone 4 to 7 divisions compared with more than
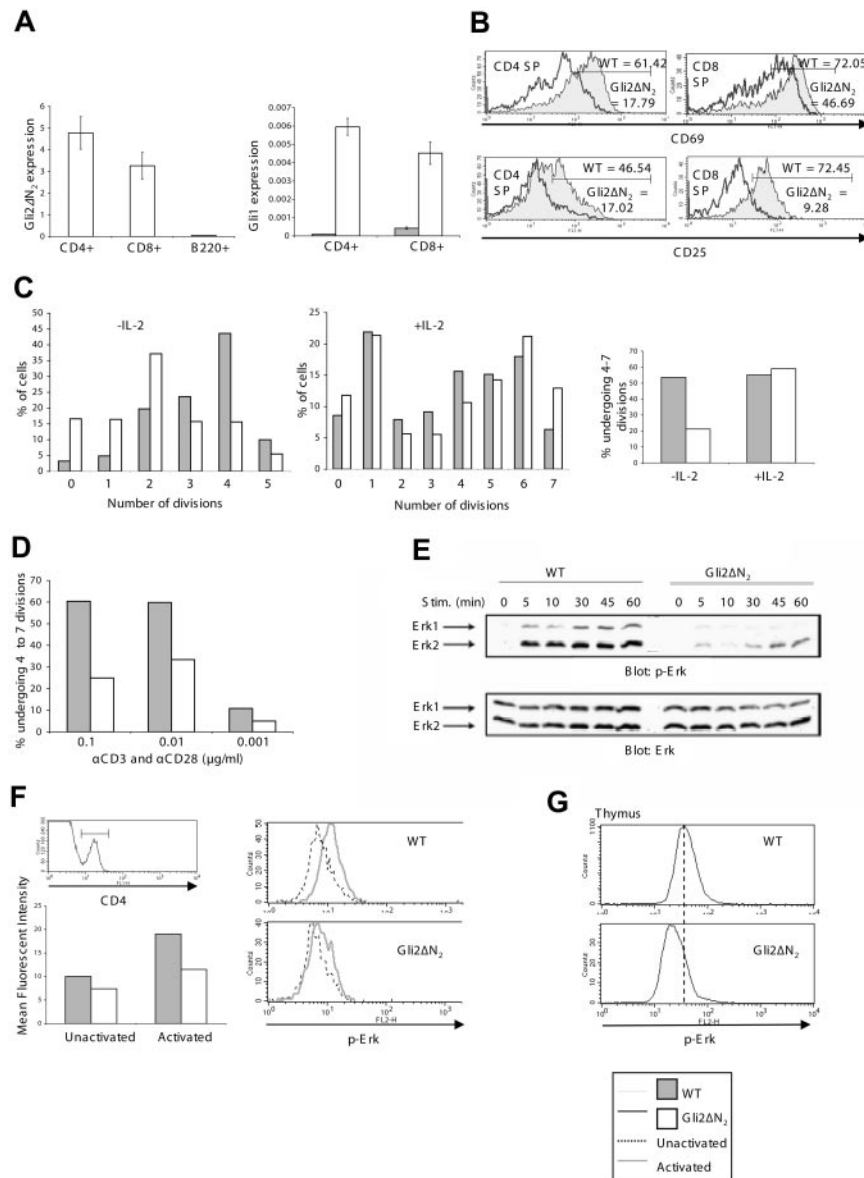
Figure 7. Gli2ΔN$_2$ expression impairs TCR-induced activation and proliferation of peripheral T cells and reduces ERK phosphorylation. (A) Quantitative RT-PCR analysis of Gli2ΔN$_2$ transgene expression in FACS-sorted peripheral T-cell populations (left graph). The right graph shows up-regulation of *Gli1* transcription on CD4 and CD8 T cells in the Gli2ΔN$_2$ (▨) compared with the WT (▢). Error bars represent SD. (B) Histograms showing the early activation marker CD69 (top panels) and the later activation marker CD25, on CD4 and CD8 SP cells after 24 hours in culture stimulated with 0.01 μg/mL of each of anti-CD3 and anti-CD28. Gray fill indicates WT; no fill, Gli2ΔN$_2$. Numbers indicate the percentage of cells falling within the marker. (In all unstimulated controls, CD69 expression was approximately 5%, and CD25 expression was approximately 8%; data not shown). (C) Bar graphs to show cell divisions using CFSE staining in CD4 cells cultured for 96 hours with 0.01 μg/mL of each of anti-CD3 and anti-CD28 with (middle panel) and without (left panel) 20 IU/mL IL-2. The numbers 0-7 represent the number of divisions for cells falling in the marker. Gray indicates WT; white, Gli2ΔN$_2$ transgenic. The bar graph on the far right shows the percentage of CFSE-labeled cells falling in the markers for 5 to 8 divisions for the WT and the Gli2ΔN$_2$ transgenic with and without IL-2. (D) Bar graph shows proliferation for WT and the Gli2ΔN$_2$ transgenic cultured in 0.1, 0.01, and 0.001 μg/mL of each of anti-CD3 and anti-CD28. (E) Western blot showing the levels of phosphorylated (upper panel) Erk1 and Erk2 kinases in WT and GliΔN$_2$ transgenic splenocytes stimulated with 1 μg/mL of each of anti-CD3 and anti-CD28 for the indicated period of time. For comparison, the total levels of Erk kinases expressed in the cytoplasm of stimulated cells is shown (lower panel). (F) Intracellular phosphorylated ERK (p-Erk) on CD4$^+$ cells. Top left histogram shows the CD4$^+$ gate. The right histograms show intracellular p-Erk levels in unactivated CD4$^+$ (dashed line) and CD4$^+$ activated with 1 μg/mL of each of anti-CD3 and anti-CD28 for 2 minutes (gray line). The bar chart shows the MFI (unactivated/activated) for WT (10.0/19.1) and GliΔN$_2$ (7.4/11.5). (G) Histograms to show basal intracellular p-Erk in WT (top, MFI = 45.2) and GliΔN$_2$ (bottom, MFI = 29.0) thymocytes.

50% of WT CD4 T cells (Figure 7C). Addition of IL-2 to the cultures overcame the inhibition of proliferation (Figure 7C). We titrated the strength of activation signal given by anti-CD3 and anti-CD28 antibodies. Although at low antibody concentration proliferation was suboptimal in the WT cultures, we consistently saw approximately 50% fewer CD4 T cells that had undergone 4 or more divisions in the Gli2ΔN$_2$ transgenic cultures compared with WT cultures (Figure 7D).

Hh signaling reduces TCR signal strength and inhibits TCR-induced ERK phosphorylation

To measure directly the effect of Hh signaling on TCR signals, we stimulated splenocytes from Gli2ΔN$_2$ transgenic and littermate mice with antibodies against CD3 and CD28 and measured MAPK activation by following the kinetics of ERK phosphorylation by Western blot. Gli2ΔN$_2$ expression inhibited TCR-induced MAPK activation (Figure 7E). In WT splenocytes, strong activation of ERK2 was observed within 5 minutes of stimulus, and full phosphorylation of ERK1 and ERK2 kinases was detected after 30 minutes. In contrast, activation of ERK2 in

Gli2ΔN$_2$ transgenic splenocytes was much delayed and reduced, and ERK1 failed to activate to normal levels during the experiment. To confirm that this reduction in ERK phosphorylation was T-cell specific, we used a FACS-based assay[27] to compare Erk phosphorylation in the CD4$^+$ T-cell population. Gating on CD4$^+$ cells, the MFI of intracellular anti–phospho-ERK on unactivated cells was slightly lower in the Gli2ΔN$_2$ compared with WT spleen. On TCR ligation, there was a clear shift in the WT intracellular anti–phospho-ERK staining, with a doubling in MFI. In contrast, the shift in the Gli2ΔN$_2$ T cells was negligible (Figure 7F). Thus, expression of the Gli2ΔN$_2$ transgene inhibited TCR-induced activation by a mechanism upstream of ERK phosphorylation.

Given that basal phospho-ERK levels were reduced in Gli2ΔN$_2$ peripheral T cells, we also measured ERK phosphorylation in thymocytes (Figure 7G). The MFI of intracellular anti–phospho-ERK was reduced, typically from 45 in WT to 29 in Gli2ΔN$_2$ thymocytes. This reduction in basal ERK phosphorylation is consistent with reduction in TCR signal strength during repertoire selection in the Gli2ΔN$_2$ transgenic.

Discussion

Here, we show that the transcriptional events induced by activation of the Hh signaling pathway in mature peripheral T cells resulted in a reduction of TCR signal strength, profoundly inhibiting the ability of the TCR to transduce signals for activation and proliferation. Likewise, activation of the Hh signaling pathway in thymocytes changed the outcome of TCR ligation at many stages of development: allowing self-reactive T cells to escape clonal deletion; reducing transgenic TCR-mediated positive selection of CD8 T cells; and reducing the ratio of CD4/CD8 SP cells produced in the thymus. In contrast, in the Shh^{−/−} thymus the CD4/CD8 ratio was affected in the opposite way, and differentiation from DP to CD4SP was increased. Both positive and negative selection of a transgenic TCR were also increased, demonstrating that Shh does indeed influence TCR repertoire selection and the transition from DP to SP cell in a physiological situation. Taken together, the consequences for thymocyte development of removal of the Shh signal (in the Shh^{−/−} thymus), or activation of Hh signaling in vivo (by Gli2ΔN$_2$ expression) and in vitro (by Shh treatment), all strongly support the hypothesis that Hh signaling in thymocytes modulates TCR signal strength.

Reduction of TCR signal strength by Hh-dependent transcription has many consequences for thymocyte development and peripheral immunity, as the outcome of TCR ligation would depend on the Hh microenvironment of the cell. Hh proteins have been shown to function as morphogens,[1,41] produced at localized sources and specifying different cell fates in a concentration-dependent manner. Thymocytes move through distinct areas of the thymus at different stages of their development,[28] and therefore will receive different strengths of Hh signal at different stages. Our data suggest that their location in the thymus, relative to the source of Hh, will influence the outcome of TCR ligation (positive or negative selection, CD4/CD8 lineage decisions). Of interest, Shh is expressed by epithelial cells scattered in the medulla and corticomedullary junction, and the thymocyte populations that are located in this part of the thymus (early DN populations and SPs) show highest expression of the Hh-target gene *Gli1* (Figure 1). As not all medullary epithelial cells express Shh,[4,6] it is possible that those that express Shh are specialized for a particular function in TCR repertoire selection.

In the periphery, Hh signaling could function to change the threshold of T-cell activation and influence TCR-dependent T-cell fate decisions, such as TH1/TH2 differentiation or the induction of anergy. Shh is expressed by follicular dendritic cells,[42] but Hh proteins are also expressed in skin, gut, and lung, where they are involved in tissue homeostasis and renewal.[1] These tissues as sites of frequent immune challenge by pathogens are also subject to many inflammatory and autoimmune diseases, and Hh expression could function to reduce the initial threshold for T-cell activation. Up-regulation of Hh proteins during renewal or remodeling after infection or tissue damage could further influence T-lymphocyte populations at sites of damage.

Inappropriate activation of the Hh signaling pathway has been implicated in many cancers,[2] and expression of Gli2ΔN$_2$ under the control of the keratin-5 promoter increased the incidence of skin tumors in mice.[43] Of interest, we have not observed T-cell tumors in Gli2ΔN$_2$ transgenic mice, although we have maintained the colony for 3 years (N.J.R., unpublished observation, February 2004 to present).

Inhibition of TCR signaling by Hh-induced transcriptional changes involves compromised MAPKinase activation (Figure 7E-G). It is unclear why addition of IL-2 restores proliferation of activated Gli2ΔN$_2$ peripheral T cells, but one possibility is that the defect in proliferation is directly due to a decrease in IL2 transcription caused by the reduction in ERK activation.[44,45] How Gli2ΔN$_2$ inhibits TCR-induced ERK phosphorylation remains to be determined, but both TCR and Hh signaling require PKA activity, which may therefore be involved in interactions between the 2 pathways.

It has previously been reported that addition of Shh to partially purified T-cell populations in vitro enhanced the induction of T-cell activation and proliferation by suboptimal concentrations of anti-CD3 and anti-CD28 antibody, whereas addition of anti-Hh antibody to these cultures reduced activation and proliferation.[19,20] In contrast, our data indicate that the Hh pathway can inhibit T-cell activation and proliferation. There are several possible explanations for this discrepancy: Gli2ΔN$_2$ transgene expression was restricted to T cells (Figure 7A), but addition of Shh to partially purified T-cell populations might affect T-cell activation indirectly through another cell type present in the culture. Alternatively, differences in the method or strength of activation of the signaling pathway may lead to differences in the relative timing of complete induction of Hh and TCR signaling.

Recently,[8] conditional deletion of Smo from T-lineage cells has failed to reveal any positive or negative influence on anti-CD3 induced T-cell proliferation in vitro. From the data presented here, we predict that Smo-deficient T cells transduce a stronger TCR signal than WT T cells, and therefore that differences in proliferation would be detectable only if a suboptimal anti-CD3 signal were given. In the same study, early Smo deletion in the thymus confirmed that Hh positively signals for survival and proliferation of early DN cells,[8] but did not influence later stages of T-cell development. Our experiments show that Hh signaling is involved at the transition from DP to SP cell and in TCR repertoire selection. We observed increased CD4/CD8 ratio and TCR repertoire selection in the Shh^{−/−} thymus after backcrossing our Shh^{+/−} colony onto a pure C57BL/6 background[5] and introducing a transgenic TCR, whereas in the conditional Smo study the mice were outbred and effects on TCR repertoire were not examined.[8]

We have previously shown that addition of Shh to FTOC negatively regulated the transition from DN to DP cell, whereas treatment with anti-Hh antibody enhanced differentiation to DP cell.[4] The Gli2ΔN$_2$ transgenic confirms that Hh can act as a negative regulator of TCR signaling, but inhibition of pre-TCR signaling for differentiation from DN to DP cell was not observed. Similarly, transgenic mice that express dominant-negative components of the MAPKinase cascade under the control of the *lck* promoter have failed to show reduced differentiation to DP cell, despite the fact that MAPKinase activation is downstream of pre-TCR signaling.[10] The reason why use of this transgenic cassette is not ideal for dissecting signaling events downstream of the pre-TCR signal is unclear, but it may reflect incomplete transgene expression in the DN subsets,[29,30] or powerful pre-TCR independent mechanisms that allow recovery of the DP population.

In summary, we have shown that that the Hh signaling pathway influences TCR repertoire selection in the thymus and can reduce the strength of the TCR signal in mature peripheral T cells, having profound effects for T-cell development and function. It will be

important to test the consequences of Hh-induced reduction in TCR signal strength in peripheral immunity and human disease.

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Authorship

Contribution: All authors contributed experimentally and in preparation of the paper.

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