The Hedgehog Receptor Patched1 in T Cells Is Dispensable for Adaptive Immunity in Mice

Kai D. Michel¹, Anja Uhmann², Ralf Dressel¹, Jens van den Brandt¹*, Heidi Hahn², Holger M. Reichardt¹*

¹ Institute for Cellular and Molecular Immunology, University of Göttingen Medical School, Göttingen, Germany, ² Institute for Human Genetics, University of Göttingen Medical School, Göttingen, Germany

Abstract

Hedgehog (Hh) signaling modulates T cell development and function but its exact role remains a matter of debate. To further address this issue we made use of conditional knock-out mice in which the Hh receptor Patched1 (Ptch) is inactivated in the T cell lineage. Thymocyte development was moderately compromised by the deletion of Ptch as characterized by reduced numbers of CD4 and CD8 single-positive cells. In contrast, peripheral T cells were not affected. Proliferation and IFNγ secretion by Ptch-deficient T cells were indistinguishable from controls irrespectively of whether we used strong or suboptimal conditions for stimulation. Analysis of CTL and Treg cell functions did not reveal any differences between both genotypes, and T cell apoptosis induced by glucocorticoids or γ-irradiation was also similar. Surprisingly, absence of Ptch did not lead to an activation of canonic Hh signaling in peripheral T cells as indicated by unaltered expression levels of Gli1 and Gli2. To test whether we could uncover any role of Ptch in T cells in vivo we subjected the mutant mice to three different disease models, namely allogeic bone marrow transplantation mimicking graft-versus-host disease, allergic airway inflammation as a model of asthma and growth of adoptively transferred melanoma cells as a means to test tumor surveillance by the immune system. Nonetheless, we were neither able to demonstrate any difference in the disease courses nor in any pathogenic parameter in these three models of adaptive immunity. We therefore conclude that the Hh receptor Ptch is dispensable for T cell function in vitro as well as in vivo.

Introduction

The Hh signaling pathway plays a critical role in development, cell fate decisions and tissue growth. Ptch, the receptor for Hh, inhibits its signaling partner Smoothened (Smo). Binding of Hh to Ptch or inactivating Ptch mutations result in derepression of Smo. This in turn triggers a cascade of downstream events which culminate in the activation of the Gli transcription factors Gli2 and Gli3, eventually leading to the expression of Hh target genes. Those include Gli1, which further amplifies the initial Hh signal at the transcriptional level, and frequently Ptch itself [1,2].

Several members of the Hh signaling pathway such as Smo, Ptch and Gli1 are expressed in T cells [3,4]. As a matter of fact, various experimental studies indicated that Hh signaling plays a crucial role in T cell development. For example, Sonic Hedgehog (Shh), the main mediator of Hh signaling, regulates differentiation from double-negative to double-positive thymocyte and controls thymocyte progenitor homeostasis [5,6,7]. In the thymus, cell-intrinsic Gli2 levels modulate the ratio of CD4 to CD8 single-positive cells [8], and stromal Gli3 expression was proposed to be involved in the differentiation of T cells [9]. In addition, we and others have identified Ptch as an exclusively T cell-extrinsic factor necessary for proper development of T cells at their prethymic stage [10,11,12].

Besides its involvement in T cell development, Hh signaling may also control the function of mature T lymphocytes. Analysis of peripheral T cells revealed that activation of CD4⁺ or CD8⁺ T cells with anti-CD3/CD28 antibodies increased the expression of Smo [3]. Addition of recombinant Shh-N enhanced the proliferative activity of T cells, in particular under suboptimal conditions [3,4,13]. It also increased production of cytokines such as IL-2, IL-4, TNFα and IFNγ elicited by the treatment with anti-CD3 antibodies and led to an upregulation of activation markers such as CD25 and CD69 [3,13]. Shh-N cooperated with anti-CD3 antibodies in enhancing cyclin A and cytokine-inducible SH2-containing protein (CIS-1) expression, thus mimicking T cell receptor costimulation [3]. In contrast, Shh-N did not augment Bcl-XL levels in anti-CD3 stimulated CD4⁺ T cells, indicating that Hh and CD28 signaling share some but not all downstream targets [3]. A possible explanation for the impact of Hh signaling on T cell proliferation came from the finding that Bcl-2 is upregulated by addition of Shh during T cell activation [4].

On the other hand, several data argue for a repressive rather than an activating function of Hh signaling in T lymphocytes. Inhibition of the Hh pathway by transgenic overexpression of the repressor form of Gli2 under the lck promoter (Gli2△C2) increased differentiation from double- to single-positive thymocytes and augmented peripheral T cell numbers [14]. The Gli2△C2
transgene also conferred hyper-responsiveness when T cells were activated by ligation with anti-CD3 and anti-CD28 antibodies [14]. *Vice versa*, constitutive activation of Hh signaling by expression of a transgenic activator form of Gli2 under the control of the lck promoter (Gli2ΔN2) inhibited T cell activation and proliferation, probably by repressing TCR signal transduction [6]. Finally, one study in which Smo was conditionally deleted from T cells failed to reveal any influence of the loss of Hh signaling on anti-CD3 induced T cell proliferation [7]. This highlights that the currently available data regarding the role of Hh signaling in T cells are highly contradictory.

We recently reported that thymocyte development was independent of T cell-intrinsic Ptch expression [12]. Using a pure C57BL/6 background we here reinvestigated the role of Ptc in T cell development and additionally analyzed its function in peripheral T cells. This was accomplished by conditionally ablating Ptc by breeding PtcΔN2/flox mice with CD4Cre transgenic mice, by analyzing thymocytes and various subsets of peripheral T lymphocytes *in vivo* and by subjecting the mutant mice to three different models of adaptive immune responses *in vivo*. However, despite a comprehensive set of assays addressing many different aspects of T cell function we were unable to identify any role of Ptc in this cell type. Our findings therefore argue that the Hh receptor Ptc, although not necessarily Hh signaling itself, does not play a major role in peripheral T cells.

**Materials and Methods**

**Animal Experimentation**

All mice were bred under SPF conditions in our animal facility in Göttingen and used at an age of 6–24 weeks. Food and drinking water were provided *ad libitum*. *PtcΔN2/flox*, *CD4Cre/-* mice were obtained by crossing *PtcΔN2/flox* mice [11] with *CD4Cre/-* transgenic mice [15], which results in the recombination of the *PtcΔN2* locus starting at the DN3 stage of thymocyte development [15]. Importantly, a T cell-specific phenotype has neither been reported for *CD4Cre/-* transgenic nor *PtcΔN2/flox* mice, which allowed us to use them as controls in our experiments [7,11,15]. For most *in vitro* and all *in vivo* experiments mice had been backcrossed to the C57BL/6 background for more than 10 generations. Genotyping was achieved by PCR using the previously described primer combinations [12]. C3H/HeN, C57BL/6 and Balb/c mice were purchased from Charles River (Sulzfeld, Germany). All animal experiments were conducted according to ethical standards of human animal care and approved by the authorities of Lower Saxony (**Nds. Landesamt für Verbraucherschutz und Lebensmittelsicherheit**, Permit Numbers: 33.9.42502-04/007/08; 33.9.42502-04/049/08; 33.14.42502-04/109/09). All efforts were made to minimize suffering of the mice.

**Flow Cytometry**

Thymocytes and splenocytes were obtained by mechanical breakup of freshly dissected organs using forceps before the cells were passed through a 40 μm nylon mesh and washed in phosphate-buffered saline (PBS) plus 0.1% BSA. Alternatively, cells were directly used after magnetic separation or cell culture. All antibodies for FACS staining were obtained from BD Biosciences (Heidelberg, Germany), BioLegend (Uithoorn, The Netherlands), eBioscience (Frankfurt, Germany) or AbD Serotech (Düsseldorf, Germany) and directed against the following antigens (clone name in parenthesis): TCRβ (H57-597), CD3ε (17A2), CD4 (RM4-5), CD8α (53-6.7), CD25 (7D4), CD44 (IM7), CD69 (H1-2F3), GTR (DTA-1), Foxp3 (FJK-16B), F4/80 (CL:A3-1), Ly-6C/G (Gr-1; RB6-8C5) and SiglecF (E50-2440). 7-AAD was purchased from BD Biosciences. The antibodies were either directly labeled with FITC, PE, PerCP, PE-Cy7, APC or APC-Cy7 or coupled to biotin, which was detected using a streptavidin-fluorochrome conjugate. Staining was performed according to standard procedures [16] and analyzed using a FACS Canto II device in combination with FACSDiva (BD Biosciences) or FlowJo (TreeStar, Ashland/OR, USA) software. Intracellular staining of Foxp3 was accomplished using the Foxp3/Transcription Factor Staining Buffer Set according to the manufacturer’s instructions (eBioscience).

**T cell Purification**

T cells were magnetically isolated from total splenocytes as previously described [17] by using the Pan T Cell Isolation Kit II in conjunction with an autoMACS separator (both from Miltenyi Biotech, Bergisch Gladbach, Germany). Cell purity was assessed by FACS analysis and was routinely around 95%. CD4+CD25+ Treg cells and CD4+CD25- Th cells used for suppression assays were purified by employing the Regulatory T Cell Isolation Kit together with an autoMACS separator as described elsewhere [18]. Cell purity was determined by FACS analysis using antibodies against TCRβ, CD4, GITR and Foxp3 and was routinely greater than 95%.

**RNA Isolation and Quantitative RT-PCR**

Total RNA was isolated from purified splenic T cells using the Quick-RNA Mouse Prep Kit (Zymo Research, Irvine, CA, USA) or from mouse embryos using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was achieved with the help of the iScript cDNA Synthesis Kit (Bio-Rad, Munich, Germany) according to the manufacturers’ instructions. For relative quantification of gene expression, qRT-PCR was performed using the 7500 Real Time PCR System in conjunction with the Power SYBR Green PCR Master Mix (both from Applied Biosystems). Detection of individual transcripts was achieved using the following primer combinations: *wt Ptc* (5'- AAA GCC GAA GTT GGC CAT GGC TAC -3’/5’- TGC TGG GGA GTC ATT AAC TGC A -3’; *PtcΔN2* (5’- AAA GCC GAA GTT GGC CAT GGC TAC -3’/5’- TTA AAC AGG CAT AGG CAA GCT GAC -3’), *PtcΔN2* (5’- TCC AAC TAT CAC TCT AGT AGA AAT G -3’/5’- TTT TCA ATC ATC AGC TCG AT -3’), *Gli1* (5’- TAC ATG CTT CGG TGG CAT CAT CCA G -3’/5’- AGG GAA GTG GCC TGT TCA GGA -3’), *Gli2* (5’- GAT CCG CAT CA GAC CACT GCA C -3’/5’- GTG TCT TCA GTC TGT CCA GGC -3’); Amplification of *Hprt1* (5’- TCT CGG TGG CCA TCT GTC TA -3’/5’- GGG CAA CAG CAA C -3’) served to normalize for the amount of cDNA in each sample. All samples were measured in duplicates and analyzed using the Sequence detection Software (Applied Biosystems).

**Apoptosis Assay**

Apoptosis of splenic T cells was induced by treatment with dexamethasone (Dex) or exposure to γ-irradiation. In brief, 2×10^6 cells were seeded in 96-well flat bottom plates and water-soluble Dex (Sigma-Aldrich, Taufkirchen, Germany) was added at escalating doses to the cultures. Alternatively, cells were exposed to different doses of γ-irradiation before culture using a RS 225 X-Ray Research System (Gulmay Medical Systems, Chertsey, Surrey, UK) operated at 130 kV, 15 mA and with a 0.5 mm Cu filtration. After 24–96 hours, cells were harvested, stained with 7-AAD and the amount of viable cells was determined by FACS analysis.
T Cell Activation and Proliferation Assay

For polyclonal T cell activation, 10^6 purified T lymphocytes were plated in 96-well flat bottom plates and stimulated for 24–72 hours either by adding ConA (Sigma-Aldrich) or soluble anti-CD3 and anti-CD28 antibodies (BD Biosciences or Biologend) in suboptimal (0.5 μg/ml and 0.01 μg/ml, respectively) or optimal (2.5 μg/ml and 1.0 μg/ml, respectively) concentrations. Cells were stimulated in 200 μl RPMI 1640 medium with Glutamax, 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin (all from Invitrogen, Karlsruhe, Germany). For quantification of IFNγ levels, an 50 μl aliquot of the supernatant was collected from each well and analyzed by ELISA using the BD OptEIA mouse IFNγ ELISA Set (BD Biosciences) according to the manufacturers’ instructions. For quantification of T cell proliferation, the cells were subsequently labeled with 3H-thymidine (Hartmann Analytics, Braunschweig, Germany) at a dose of 37 kBq/well and cultured for another 16 hours. The labeled DNA was collected onto Filtermat A glassfibre filters using a MicroBeta 37 kBq/well and cultured for another 16 hours. The labeled DNA was collected onto Filtermat A glassfibre filters using a MicroBeta 2 ß-scintillation counter (all Perkin Elmer, Rodgau, Germany).

Suppression Assay

The suppressive capacity of T<sub>reg</sub> cells was determined essentially as described [18]. Conventional CD4<sup>+</sup>CD25<sup>+</sup> Th cells (10<sup>6</sup> cells/well) were cultured in RPMI 1640 medium with Glutamax supplemented with 10% FCS and antibiotics in 96-well U-bottom plates with different ratios of syngeneic CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells. Both cell types were either purified from Ptch<sup>lox/lox</sup> or Ptch<sup>lox/lox</sup>CD4Cre<sup>+/+</sup> mice. Polyclonal activation was achieved by adding 1 μg/ml soluble anti-CD3 and 5 μg/ml anti-CD28 antibodies into the cultures. Th or T<sub>reg</sub> cells alone (stimulated with anti-CD3 and anti-CD28) or unstimulated Th cells served as positive and negative controls. After 48 hours, supernatants were collected and IL-2 levels were assessed by using the BD OptEIA mouse IL-2 ELISA Set (BD Biosciences) according to the manufacturer’s instructions.

Cytotoxicity Assay

Alloreactive CTLs were generated by intraperitoneal immunization of Ptch<sup>lox/lox</sup> and Ptch<sup>lox/lox</sup>CD4Cre<sup>+/+</sup> mice (H<sub>2</sub><sup>b</sup>) with 2 x 10<sup>6</sup> splenocytes obtained from C3H/HeN (H<sub>2</sub><sup>a</sup>) mice. The immunization was repeated twice with an interval of 10 days. Another 10 days later the splenocytes were harvested and restimulated in vivo for 5 days by co-culturing 7.5 x 10<sup>5</sup> responder cells with 7.5 x 10<sup>5</sup> irradiated (25 Gy) splenocytes from C3H/HeN (H<sub>2</sub><sup>b</sup>) mice. Coculture was done in 96-well U-bottom plates in RPMI 1640 medium with Glutamax, 10% antibiotic water (25 μg/ml neomycin) for four weeks. Mice were monitored every other day for survival and their health status was assessed according to five clinical parameters (posture, activity, fur ruffling, diarrhea and weight loss) as described [17], each of which received a score from 0 to 2, resulting in a total score between 0 and 10. Due to ethical reasons, mice were sacrificed when the total clinical score exceeded a value of 7 for more than one day.

Measurement of Ig Serum Levels by ELISA

MaxiSorp flat bottom 96-well plates (Nunc GmbH, Langenselbold, Germany) were coated over night at 4°C with 50 μg/ml ovalbumin (Ova) in coating buffer (0.1 M sodium carbonate, pH = 9.5). The wells were washed with 0.05% Tween-20 in PBS and blocked with 10% FCS in PBS for 1 hour at room temperature. Subsequently, serum samples were added over night at 4°C. For IgE detection, the serum was initially incubated with Protein G PLUS agarose (Santa Cruz Biotechnology, Santa Cruz, CA, USA) to remove IgG antibodies, centrifuged and only the IgG-free supernatant was used. Ova-specific immunoglobulins were detected using HRP-coupled anti-IgG1, anti-IgG2a and anti-IgE specific antibodies (Southern Biotech, Birmingham, AL, USA). The reaction was quantified by measuring the absorbance at 450 nM and 570 nm using a PowerWave 340 ELISA reader (BioTek, Winooski, VT, USA).

Graft-versus-host Disease (GvHD) Mouse Model

To induce an acute GvHD reaction, 8–10 weeks old male Balb/c recipient mice received 10<sup>7</sup> T cell-depleted syngeneic bone marrow cells plus 2 x 10<sup>6</sup> T cells from Ptch<sup>lox/lox</sup>CD4Cre<sup>+/−</sup> or Ptch<sup>lox/lox</sup> control donor mice one day after irradiation with 8.5 Gy. In detail, bone marrow was isolated from femurs of C57BL/6 mice and T cell depletion was achieved using anti-CD90.2 microbeads and an autoMACS separator (both from Miltenyi Biotech) according to the manufacturer’s instructions. Cell purity was assessed by FACS and revealed that T cell contamination was routinely less than 1%. T cells were isolated from spleens and cervical, mesenteric and inguinal lymph nodes of Ptch<sup>lox/lox</sup>CD4Cre<sup>+/−</sup> and Ptch<sup>lox/lox</sup> control C57BL/6 mice as described before [17]. Bone marrow with or without (control) T cells was mixed and injected in a total volume of 200 μl PBS into the tail vein. Starting from one day before cell transfer, mice were kept on antibiotic water (25 μg/ml neomycin) for four weeks. Mice were monitored every other day for survival and their health status was assessed according to five clinical parameters (posture, activity, fur ruffling, diarrhea and weight loss) as described [17], each of which received a score from 0 to 2, resulting in a total score between 0 and 10.

Allergic Airway Inflammation Mouse Model

Female Ptch<sup>lox/lox</sup> and Ptch<sup>lox/lox</sup>CD4Cre<sup>+/−</sup> mice were immunized on days 0, 9 and 18 by intraperitoneal injections of 10 μg chicken egg ovalbumin (Ova) in coating buffer (0.1 M sodium carbonate, pH = 9.5). The wells were washed with 0.05% Tween-20 in PBS and blocked with 10% FCS in PBS for 1 hour at room temperature. Subsequently, serum samples were added over night at 4°C. For IgE detection, the serum was initially incubated with Protein G PLUS agarose (Santa Cruz Biotechnology, Santa Cruz, CA, USA) to remove IgG antibodies, centrifuged and only the IgG-free supernatant was used. Ova-specific immunoglobulins were detected using HRP-coupled anti-IgG1, anti-IgG2a and anti-IgE specific antibodies (Southern Biotech, Birmingham, AL, USA). The reaction was quantified by measuring the absorbance at 450 nM and 570 nm using a PowerWave 340 ELISA reader (BioTek, Winooski, VT, USA).
available upon request). Lungs were incubated at room temperature in Roti-Histofix 4% (Carl Roth GmbH, Karlsruhe, Germany) over night before they were used for histological analysis. For serum analysis, blood was collected from the vena renalis and centrifuged; the serum was then collected and frozen for later analysis. Erythrocytes were lysed using a haemolysis buffer (400 μM Tris, 155 mM ammonium chloride, pH = 7.2). The cells were plated in 96-well flat bottom plates and stimulated with 10 μg/ml Ova for 72 hours. Non-stimulated or ConA-stimulated cells served as controls. Proliferation and cytokine production were assessed as described above.

Melanoma Mouse Model

The previously published B16-F10 murine melanoma cells were kindly provided by Dr. Jürgen Becker (Clinic of Dermatology, University of Würzburg, Germany) [20]. The cells were grown in RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. For tumor vaccination, cells were harvested, washed twice in PBS, irradiated with 125 Gy and mixed at a ratio of two volumes of cells to one volume of complete Freund’s adjuvant (CFA; Sigma-Aldrich). Subsequently, 10^5 cells were injected in a total volume of 150 μl subcutaneously into the left flank. Ten days after immunisation, mice were challenged with 10^4 live B16-F10 cells into the right flank. Starting from day 7 after challenge, mice were regularly monitored for palpable tumors. Tumor size was recorded from two caliper measurements of the longest (a) and shortest (b) diameter and the tumor volume was calculated according to the following formula: volume = a × b^2 × 0.4 [21]. The experiment was terminated for individual mice when the tumor volume exceeded 800 mm^3 or when ulceration or bleeding occurred.

Statistical Analysis

Statistical analysis was performed by unpaired t-test, Mann Whitney or log-rank test, and the data are depicted as mean ± SEM. For serum analysis, blood was collected from the vena cava into an EDTA tube. Serum was isolated by centrifugation and stored at -80°C. For cytokine analysis, sera were diluted 1:100 in RPMI medium supplemented with 10% FCS and 1% penicillin/streptomycin. The concentrations of cytokines were measured by ELISA. For cell proliferation assay, cells were seeded in 96-well flat bottom plates and stimulated with 10 μg/ml ConA (Invitrogen). The cells were pulsed with [3H]-thymidine (2 μCi, 100 μl, 1 mCi/mM, NEN) for 6 hours before they were harvested using a cell harvester (Skatron). Proliferation was determined by counting incorporated [3H]-thymidine using a beta-counter (Wallac). The mean ± SEM of four mice of each genotype is shown. Statistical analysis was performed by unpaired t-test (*: p<0.05). Differences were not statistically different unless otherwise indicated. doi:10.1371/journal.pone.0061034.g001

Results

Disruption of Ptch in the T Cell Lineage Moderately Impacts Thymocyte Development while Peripheral T Lymphocytes are Unaffected

We previously reported that T cell-specific deletion of Ptch did not impact thymocyte development in Ptch^fl/fl*CD4Cre^+/− mice [12]. As our findings were in contrast to several other reports describing an influence of Hh signaling on the transition from the double-positive (DP) to the single-positive (SP) stage of thymocyte development [22,23,24], we decided to repeat the analyses with both genotypes, we found that the relative number of DP thymocytes was increased in Ptch^fl/fl*CD4Cre^+/− mutant mice while the percentages of CD4 and CD8 SP thymocytes were decreased (Figure 1C). Even though the magnitude of these differences was small, they nevertheless reached statistical significance and were qualitatively in line with previous reports [22,23,24]. This encouraged us to ask whether the deletion of Ptch would also affect peripheral T cells.

In our previous work we demonstrated that recombination of the Ptch locus and the resulting expression of the non-functional Ptch^{Δ/Δ} allele were almost complete in thymocytes of Ptch^{Δ/Δ} mice [12]. Nonetheless, this did not lead to an upregulation of the two target genes Gli1 and Gli2 as one would have predicted for activated canonical Hh signaling [12]. Therefore we performed the same analysis for peripheral T cells by isolating splenocytes from mice of both genotypes followed by qRT-PCR analysis. Surprisingly, we again found that disruption of Ptch was almost complete in mutant T cells and that this had no effect on Gli1 and Gli2 expression (Figure 2A). We also analyzed expression of Ptc2 to determine whether this highly homologous protein might compensate for the loss of Ptch. Importantly, we could not detect any Ptc2 mRNA in T cells of either genotype while Ptc2 was abundantly expressed at embryonic stage E10.5.

Next we studied the cellular composition of peripheral lymphoid organs. Size and cellularity of the spleen were unaltered in Ptch^fl/fl*CD4Cre^+/− mutant mice and the same was true for the percentages of splenic T cells as well as of CD4+ and CD8+ cells amongst them (Figure 2B,C). Similar observations were made for lymph nodes and peripheral blood (data not shown). To unravel a potentially more subtle impact of the ablation of Ptch, we enumerated recently activated T cells based on CD69 surface expression as well as the CD44hi memory T cells. Nonetheless, the percentages of both populations were similar amongst the CD4+ or CD8+ T cells in Ptch^fl/fl*control and Ptch^fl/fl*CD4Cre^+/−.
mutant mice irrespective of whether we analyzed spleen, lymph nodes or blood (Figure 2D and data not shown). We conclude that the deletion of Ptch in the T cell compartment does not impact the composition of peripheral T cells.

Ablation of Ptch does not Impact Polyclonal T Cell Activation

To explore the role of Ptch for T cell activation, we sorted splenic T lymphocytes and stimulated them either with anti-CD3/CD28 antibodies or Concanavalin A (ConA). Since Hh signaling
was previously reported to be particularly relevant under suboptimal stimulation conditions, anti-CD3/CD28 and ConA were used at two different concentrations. T cell proliferation and IFNγ production were monitored over a 72 hours period by 3H-thymidine incorporation assay and ELISA, respectively. When we stimulated the T cells under optimal conditions (1.0 μg/ml anti-CD3/CD28 or 2.5 μg/ml ConA), proliferation of Ptchfloxed/floxed and Ptchfloxed/floxed CD4Cre+/- cells was strong but similar at any time point (Figure 3A). When we used anti-CD3/CD28 or ConA at suboptimal concentrations (0.01 μg/ml and 0.5 μg/ml, respectively), proliferation was overall much weaker but ablation of Ptch had again no effect (Figure 3A). Unlike activated T cells, resting T lymphocytes from control and Ptch-deficient mice hardly proliferated at all (data not shown). IFNγ secretion increased over time under optimal stimulation conditions, while it was very low under suboptimal conditions. Nonetheless, there was no difference between both genotypes (Figure 3B). We conclude that T cell activation is unaffected by the absence of Ptch both under optimal and suboptimal stimulation conditions.

Ptch has No Impact on the Cytolytic Capacity of CTLs

We also determined the role of Ptch for the effector function of CD8+ T cells by analyzing the cytolytic capacity of alloreactive CTLs (Figure 4A). Ptchfloxed/floxed control and Ptchfloxed/floxed CD4Cre+/- mutant mice (H2b) were repeatedly immunized with allogeneic splenocytes from C3H/HeN (H2k) mice. Subsequently, the splenocytes of the recipients were harvested, restimulated in vitro with splenocytes from C3H/HeN mice and subjected to a chromium release assay using Ltk- target cells (H2k). The specific lysis of these targets cells by mutant and control CTLs was similar (p = 0.216 by 2-way ANOVA), indicating that Ptch was not required for the cytolytic function of CTLs in vitro.

The Abundance and Suppressive Capacity of Treg Cells is Independent of Ptch

To determine the abundance of naturally occurring Treg cells in the spleen we performed a flow cytometric analysis. Approximately 8% of all CD4+ T cells in Ptchfloxed/floxed control mice were CD25+GITR+FoxP3+ Treg cells and their frequency was similar in mutant Ptchfloxed/floxed CD4Cre+/- mice (Figure 4B, left panel). Given

Figure 3. Proliferation and IFNγ production by activated T cells. Total T cells were isolated from the spleens of Ptchfloxed/floxed control or Ptchfloxed/floxed CD4Cre+/- mutant mice and 10^5 cells per well were stimulated with different concentrations of ConA or anti-CD3/CD28 antibodies in a total volume of 200 μl medium. For detection of IFNγ, 50 μl medium were removed at each time point and used for analysis by ELISA. For measurement of proliferation, an equal volume of fresh medium containing 37 kBq 3H-thymidine was added to the same wells and incubated for another 16 hours. (A) Proliferation was assessed by scintillation counting. (B) Quantification of IFNγ levels was achieved by ELISA. Each panel shows the mean ± SEM of five individual experiments. Statistical analysis for each experimental condition was performed by unpaired t-test and no differences were found in any case.

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Deletion of Ptch has No Impact on T Cell Apoptosis

Synthetic glucocorticoids such as dexamethasone (Dex) and DNA-damage caused by γ-irradiation are known to induce T cell apoptosis by activating the pro-apoptotic proteins Bim and Puma [25]. To assess the role of Ptch in this process, we sorted T cells from the spleens of Ptch$^{flox/flox}$ control or Ptch$^{flox/flox}$ CD4Cre$^{-/-}$ mutant mice. Spontaneous cell death was indistinguishable between both genotypes. When T cells were γ-irradiated with a dose of 1 or 2 Gy and cultured for up to 4 days, we observed a dose- and time-dependent increase of cell death which was indistinguishable between both genotypes (Figure 5A). In another setting, we cultured T cells in the presence of either 2 nM or 6 nM Dex for up to 4 days. There was a dose- and time-dependent increase of apoptosis which was again unaltered in Ptch-deficient cells (Figure 5B). We also tested whether activated T cells may exhibit any difference concerning their sensitivity to apoptosis. Following activation by anti-CD3/CD28 antibodies for 24 hours the T cells were much more resistant to Dex-induced apoptosis as compared to naïve T cells, and not affected by the treatment unless the dose was considerably increased (Figure 5B,C). None-theless, activated T cells still underwent apoptosis in the presence of 10$^{-6}$ M Dex, but there was no difference between cells isolated from Ptch$^{flox/flox}$ control or Ptch$^{flox/flox}$ CD4Cre$^{-/-}$ mice in this respect (Figure 5C). We conclude that ablation of Ptch does not impact apoptosis sensitivity, neither of resting nor activated peripheral T cells.

Adaptive Immune Responses do not Require Ptch Expression in T Lymphocytes

Since we had not observed any major difference in T cell function in vitro, we wondered whether Ptch deletion might only be relevant under more physiological conditions in vivo. To address this question, we initially analyzed a mouse model of graft-versus-host disease (GvHD), which depends on the function of both Th cells and CTLs. In humans, GvHD occurs after transplantation of a MHC mismatched bone marrow graft containing mature alloreactive T cells. In our experimental model, lethally irradiated...
Balb/c mice were transplanted with bone marrow and purified T cells from either Ptc1flox/flox control or Ptc1flox/flox CD4Cre+/- C57BL/6 mice. Upon encountering the allogeneic MHC molecules, the transferred T cells attack the host cells and induce a strong adaptive immune response dominated by Th1 cells. This leads to the occurrence of several typical disease symptoms, and eventually the mice succumb to death. Importantly, disease severity and mortality were similar in mice of both genotypes as confirmed by statistical analysis (Figure 6A,B). This indicates that Ptc1 in T cells does not affect any T cell effector functions required for the occurrence of GvHD.

In another approach we studied allergic airway inflammation, which is an experimental model of human asthma and represents a prototypic Th2 immune response. Ptc1flox/flox control and Ptc1flox/flox CD4Cre+/- mice were repeatedly immunized with ovalbumin in adjuvant and subsequently challenged intranasally to induce an inflammatory response in the lung. Compared to control mice, intranasal ovalbumin application led to a strong infiltration of leukocytes into the lung (Figure 7A,C). The bronchoalveolar

Figure 5. Sensitivity of T cells to apoptosis induction. (A) Apoptosis induction by γ-irradiation was assessed by culturing 2 × 10⁵ T cells following exposure to a dose of 1 or 2 Gy or without any manipulation. Cell viability was assessed every 24 hours by FACS analysis using 7-AAD. Results represent the mean ± SEM of three independent experiments. (B) Glucocorticoid-induced apoptosis was investigated by culturing 2 × 10⁵ splenic T cells from Ptc1flox/flox control or Ptc1flox/flox CD4Cre+/- mutant mice in medium with or without 2 or 6 nM of water-soluble Dex for 4 days. Cell survival was determined as described above. Results represent the mean ± SEM of three independent experiments. (C) T cells were pre-activated with 1.5 μg/ml soluble anti-CD3 and anti-CD28 for 24 hours. Apoptosis was then induced by adding 1 μM of water-soluble Dex and cell viability was assessed up to four days after apoptosis induction similar as above. Results represent the mean ± SEM from three Ptc1flox/flox or four Ptc1flox/flox CD4Cre+/- animals. Based on the analysis by unpaired t-test, apoptosis was not different between both genotypes. doi:10.1371/journal.pone.0061034.g005

Figure 6. Morbidity and mortality after GvHD induction. GvHD was induced in irradiated Balb/c mice (8.5 Gy) by transferring 10⁷ T cell-depleted syngeneic bone marrow cells plus 2 × 10⁶ T cells from either Ptc1flox/flox control or Ptc1flox/flox CD4Cre+/- mutant C57BL/6 donor mice (n = 29 for each genotype). Control animals received T cell-depleted bone marrow only (n = 13). Mice were monitored every other day for clinical symptoms (A) and survival (B). The figures show the combined data of five independent experiments. Statistical analysis of the disease courses was performed by Mann Whitney test; in the case of the survival curves the log-rank test was employed. In both cases, no statistical significant difference between both genotypes was found. doi:10.1371/journal.pone.0061034.g006
lavage fluid (BALF) was dominated by eosinophils but also contained CD4+ T cells, macrophages and neutrophils (Figure 7B). Nonetheless, neither the absolute numbers of the infiltrating cells nor their relative percentages were significantly different between both genotypes (Figure 7A-C). We also checked the titers of ovalbumin-specific antibody isotypes. The predominant one found in the serum was IgG1 with smaller amounts of IgG2a and IgE (Figure 7D), a finding which is typical for an allergic immune response induced in C57BL/6 mice. However, the antibody titers of all three isotypes were not significantly different between Ptch+/+ control and Ptch−/− CD4Cre+/− mice. The proliferative response of splenic T cells restimulated with ovalbumin in vitro was increased in immunized mice but to a similar extent in both genotypes (Figure 7E). Thus, we did not find any indication that the allergic immune response in a model of asthma was influenced by the presence of Ptch in T cells.

The Tumor Surveillance Capacity of the Immune System is Unaffected by Ptch Ablation in T Cells

Although we had not observed any effect of Ptch deletion on CTL and Treg cell function, we considered it possible that a potential role in these T cell subpopulations might only become evident in vivo. One function of the immune system in which both cell types are important is tumor surveillance. Whereas CTLs are one of the major effector cell types responsible for the lysis of neoplastic cells, Treg cells are known to be tumor promoting. To address this issue, we subcutaneously inoculated Ptch+/+ control and Ptch−/− CD4Cre+/− mice with B16-F10 melanoma cells and monitored tumor incidence and size over a four weeks period. However, we did not detect any differences neither in tumor progression nor size (Figure 8A). We also immunized the mice prior to tumor inoculation by using inactivated tumor cells aiming at eliciting a protective anti-tumor response. This procedure indeed decreased tumor incidence, time of onset and tumor size, but to the same extent in both genotypes as revealed by statistical analysis (Figure 8B). We therefore conclude that Ptch in T cells is dispensable for proper tumor surveillance by the adaptive immune system.

Discussion

Stromal expression of the Hh receptor Ptch plays a critical role in pre-thymic T cell development [11,12], yet it is unknown whether it also fulfills an intrinsic function in peripheral T cells. In fact, all major components of the Hh signaling pathway are expressed in T lymphocytes and, according to the current view, at least Hh and Gli2 are required for proper T cell function. Our current data support a role of the Hh receptor Ptc in intermediate and late thymocyte development but argue against an important function in peripheral T cells both as vitro and in vivo.

We employed a mouse model in which Ptc was specifically inactivated in T cells by Cre recombinase expressed under the control of the CD4 promoter, which becomes active at the DN3 stage of thymocyte development [15]. Using this strategy we found that Ptc inactivation, despite being highly efficient, affected thymocyte development only moderately. This finding was distinct from our earlier data showing that T cell development was completely independent of T cell-intrinsic Ptc expression [12]. The discrepancy could be explained by the different genetic backgrounds of the mouse strains used in both studies. Here we almost exclusively analyzed mice backcrossed to C57BL/6 for more than 10 generations, while we had previously used mice on a C57BL/6 background.

The alterations in thymocyte development that we observed here were qualitatively similar to those reported to occur in response to activated Hh signaling [22,23,24]. Importantly, however, our analyses did not reveal any impact of the deletion of Ptc on the number and phenotype of peripheral T cells. In vitro analysis demonstrated that functional characteristics of conventional T cells such as proliferation and cytokine production in response to polyclonal stimulation were nearly unaltered in Ptc-deficient T cells. Of note, the proliferation rate of polyclonally activated Ptc deficient T cells was slightly higher as compared to control cells, but this difference only emerged under highly unphysiological conditions and was insignificant at any time point. We were also unable to see differences when assessing the susceptibility of either resting or preactivated T cells to apoptosis induction. Functional analysis of CTL and CD4+ CD25FoxP3+ Treg cells in vitro did not reveal any impact of Ptc alation on these cell types either. However, we cannot exclude that other lymphocyte populations such as induced Treg cells might be affected by the absence of Ptc.

Since more subtle effects in T lymphocytes might only become evident in vivo, we used three models of adaptive immune responses addressing different aspects of T cell function. Nevertheless, we were unable to identify an impact of Ptc alation in any of these settings.

Surprisingly, there was no evidence of activated canonical Hh signaling when we analyzed target gene expression in T cells from Ptch+/+ CD4Cre−/− mice. Both Gli1 and Gli2 levels were unchanged despite successful Ptc deletion. This is consistent with our previous data [12] and could be explained by the lack of primary cilia in cells of the hematopoietic lineage such as T cells [26]. Primary cilia are microtubule-based organelles that protrude from the surface of most vertebrate cells and fulfill crucial roles in vertebrate development by providing hubs for the transduction of various developmental signaling pathways including Wnt, FGF, PDGF and also Hh [27]. Although the interaction between Hh signaling and the primary cilium is currently not fully understood, it has recently become evident that the Hh pathway is strictly coupled to this cellular compartment [28]. Therefore, the lack of primary cilia may prevent Ptc-mediated activation of Hh signaling in T cells. This hypothesis is indirectly supported by work showing that the deletion of Ptc in hematopoietic stem cells also failed to activate canonical Hh signaling [10]. Alternatively, compensatory mechanisms might account for the canonical pathway not being activated by Ptc alation. As a potential candidate mechanism, we investigated expression of the Ptc homolog Ptc2, which fulfills distinct roles from Ptc [29] but might still be able to compensate for its lack in selected cell types. However we were unable to detect Ptc2 transcripts both in Ptc deficient and control T cells. Yet the existence of other compensatory mechanisms still has to be elucidated.

Previous studies by other groups had pursued different approaches to investigate the role of Hh signaling in T cells. Initial work focused on the in vitro response of human and murine (C57Bl/6) T cells to exogenous Shh. It was reported that addition of Shh enhanced T cell activation and proliferation induced by optimal or suboptimal concentrations of anti-CD3 and anti-CD28 antibodies whereas addition of an anti-Hh antibody to these cultures reduced activation and proliferation [3,4,13]. These changes were accompanied by an increased expression of the activation-dependent cell surface markers CD25 and CD69 and an enhanced secretion of cytokines such as IL-2 and IFN-γ. Considering the current model of Hh signaling and our own findings, these results are difficult to explain. It is possible that addition of Shh to partially purified T cell populations activated...
these cells indirectly through other cell types present in the culture, or alternatively that Shh activated a non-canonical signaling pathway. It is also noteworthy that due to the artificial nature of in vitro experiments, the results may not reflect the physiological situation.

In another approach, Rowbotham and colleagues utilized transgenic mouse models on a C57Bl/6 background that either

Figure 7. Lung infiltration, antibody production and T cell function after induction of allergic airway inflammation. Ptch1 control and Ptch1\textsuperscript{floxFlox} CD4Cre\textsuperscript{+/+} mutant C57BL/6 mice were sensitized against Ova by repeated intraperitoneal injection of antigen plus adjuvant. Control mice (Ptch1\textsuperscript{floxFlox}) received adjuvant without Ova. Antigen challenge was achieved by intranasal delivery of dissolved Ova for three consecutive days and analysis was performed after a two day resting phase. (A) Lungs were washed \textit{in situ} and total cell counts in the bronchoalveolar lavage fluid (BALF) were determined using light microscopy. (B) Identification of different populations of lung-infiltrating cells within the BALF by using FACS. (C) Lung histology of mice 48 hours after the last challenge. A control lung obtained from a non-sensitized mouse challenged with Ova is shown along with lungs of Ptch1\textsuperscript{floxFlox} and Ptch1\textsuperscript{floxFlox} CD4Cre\textsuperscript{+/+} mice which were both sensitized and challenged with antigen. No pathohistological signs of inflammation could be detected in control mice whereas a clear and massive cell influx was seen in sensitized and challenged mice. (D) Serum concentrations of Ova-specific IgG1, IgG2a and IgE immunoglobulins were quantified using ELISA and are depicted in the form of optical densities (OD). (E) Splenocytes were isolated and restimulated \textit{ex vivo} with 10 μg/ml Ova for 72 hours and proliferation was assessed by \textsuperscript{3}H-thymidine incorporation assay. Results represent the mean ± SEM from nine non-sensitized control mice as well as thirteen Ptch1\textsuperscript{floxFlox} and fourteen Ptch1\textsuperscript{floxFlox} CD4Cre\textsuperscript{+/+} animals, respectively. Statistical analysis of all parameters was performed by unpaired t-test and no differences were found in any case. doi:10.1371/journal.pone.0061034.g007
expressed the transcriptional activator (Gli2ΔN2) or repressor (Gli2ΔC2) form of Gli2 to study the effect of constitutive activation or repression of Hh signaling in T cells, respectively [8,14]. They found that activation of the Hh signaling pathway exerted a negative impact on TCR signal strength with implications for positive and negative selection in the thymus and the function of peripheral T cells. Although the data obtained in both mouse models were consistent, the results need to be carefully evaluated in view of the design of the employed experimental system. Both models relied on the overexpression of artificial forms of the transcription factor Gli2 and due to this constraint, the obtained data does not necessarily reflect the physiologic function of this protein. Another group focused on the role of Hh signaling in T cells by inactivating the signal transducer Smo at different time points during T cell development [7]. They found that an early shutdown of Smo at the pro-T cell stage led to thymic atrophy, which was associated with a substantial decrease in thymocyte and peripheral T cell numbers. In contrast, a deletion of Smo in a later stage of T cell development had no effect, neither on thymocyte numbers nor subtype distribution, suggesting that Hh signaling was essential for proliferation of early thymocytes while it becomes dispensable after pre-TCR expression. These findings are in line with the current model of Hh signaling as well as our own data. It is widely accepted that most vertebrate cells including stem cells possess primary cilia [30] while hematopoietic cells lose this compartment upon maturation. It is therefore conceivable that cells may also become refractory to Hh signaling once they are committed to the hematopoietic lineage. According to the findings of El Andaloussi et al. [7], the time window during which T cells lose responsiveness to Hh is the DN stage of thymocyte development. This suggests that canonical Hh signaling via Ptch is dispensable for all subsequent steps of T cell development as well as the function of peripheral T lymphocytes.

Collectively, our studies demonstrate that the Hh receptor Ptch is neither required for peripheral T cell function in vitro nor in vivo. We propose that this is either due to the lack of the primary cilia which is required for signal initiation in most cell types, or yet elusive compensatory mechanisms or a consequence of non-canonical Hh signaling. Therefore, it is unlikely that Ptch, but not necessarily Hh signaling itself, plays an essential role in peripheral T cell function and adaptive immunity.

Figure 8. Tumor incidence and size after inoculation of B16 melanoma cells. C57BL/6 mice were inoculated with 10⁴ B16-F10 melanoma cells into the right flank and tumor growth was monitored over a period of three weeks. The incidence of palpable tumors (left panel) and the mean tumor size ± SEM (right panel) are depicted for both experimental setups. (A) Results of tumor incidence and size are shown for eleven Ptchfox/fox control and nine Ptchfox/fox CD4Cre⁺/⁻ mutant C57BL/6 mice in which tumorigenesis had been induced without additional manipulation. (B) To generate protective immunity, C57BL/6 mice were vaccinated with 10⁶ irradiated B16-F10 cells together with adjuvant subcutaneously into the left flank. Ten days later the vaccinated mice were challenged with 10⁴ viable B16-F10 cells subcutaneously into the right flank. Tumor incidence rate and mean tumor size ± SEM are shown for fourteen Ptchfox/fox and Ptchfox/fox CD4Cre⁺/⁻ mice each. Statistical analysis of tumor incidence and size was performed by Mann Whitney test and not found to be different between both genotypes.

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Author Contributions

Conceived and designed the experiments: KDM AU HH HMR. Performed the experiments: KDM AU RD JvdB. Analyzed the data: KDM AU RD JvdB HMR. Wrote the paper: KDM HH HMR.