Sonic hedgehog promotes CD4+ T lymphocyte proliferation and modulates the expression of a subset of CD28-targeted genes

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Abstract

Sonic hedgehog (Shh) is a crucial morphogen in the development of numerous tissues and organs, including the nervous system, gastrointestinal tract and lung. Recent findings suggest that Shh plays an important role in thymocyte development and peripheral T cell function. Here we report that the Shh receptors, patched and smoothened, are expressed in resting and activated T cells and their expression is regulated upon T cell activation. Shh protein is also detected on the surface of freshly isolated T cells. Although exogenous Shh alone does not activate resting T cells, it exhibits co-stimulatory activity which is reflected in its ability to potentiate CD3-mediated proliferation and cytokine production by CD4+ T cells. The co-stimulatory effect is most prominent at sub-optimal TCR stimulation level. Gene expression analysis reveals that Shh signaling in CD4+ T cells modulates a different set of transcriptional targets from that in neuronal cells. Furthermore, Shh co-stimulation modulates the expression of a subset of CD28-responsive genes, including cyclin A and B cell translocation gene 2.

Introduction

The hedgehog (Hh) family consists of highly conserved proteins that mediate intercellular signaling and it plays an important role in cell differentiation and cell patterning in a wide variety of tissues (1, 2). Hh was first characterized in Drosophila in which mutations of this protein caused disruption in larval segmentation (3). Three mammalian Hh proteins have been identified and among which sonic hedgehog (Shh) is the most extensively characterized. Shh is synthesized as a 45-kDa precursor protein and is cleaved auto-catalytically to yield two fragments with distinct functions. The sonic hedgehog 20-kDa NH2-terminal domain (Shh-N) possesses all the signaling capabilities while the sonic hedgehog 25-kDa COOH-terminal domain (Shh-C) is responsible for the auto-processing of the protein. Shh-N purified from cell extracts has undergone palmitoylation and cholesterol modification (4, 5). The diffusible form of Shh exists as a multimeric complex and recent findings have revealed that the lipid modifications not only increase its biological potency but also regulate the extracellular transportation and target cell binding of the Shh-N (6–8). The binding receptor for Shh is a 12-pass transmembrane protein, patched (Ptc). Ptc is a suppressor of another seven-pass transmembrane protein, smoothened (Smo), which mediates the signaling events induced by Shh. Upon Shh binding to Ptc, Smo is released from the inhibition and it activates transcription through the Gli zinc finger transcription factors (9).

Shh is critical for many mammalian developmental processes, including limb morphogenesis, central nervous system development and formation of the gastrointestinal tract (10–12). Perturbation of Shh signaling pathway is also associated with pathological conditions such as tumor formation and chronic lung fibrosis (13–16). Bhardwaj et al. (17) demonstrated a functional role for Shh signaling in the proliferation and differentiation of human hematopoietic stem cells.
cells through its effects on bone morphogenetic protein 4. Recent studies have also shown that Shh contributes to the development of the immune system and regulation of peripheral immunity. In T cell development, it has been reported that Shh signaling is involved in thymocyte transition from CD44+/CD25− (DN1 stage) to CD44+/CD25+ (DN2 stage) and from CD4−/CD8− DN to CD4+/CD8+ DP stages (18, 19). In addition, Shh can modulate mature T cell functions through the regulation of cell cycle progression (20, 21) and protect germinal center B cells from Fas-mediated apoptosis (22). Furthermore, Shh expression is up-regulated in certain pathological conditions in which cells of the immune system are implicated. For example, in chronic lung fibrosis, Shh is up-regulated in fibrotic epithelial cells and Ptc1 is detected on infiltrating monocuclear cells, alveolar macrophages as well as in circulating T cells (16). In a mouse model of medulloblastoma, Shh up-regulation in cerebellar granule neurons is the result of IFN-γ produced by the infiltrating CD3+ T cells (15). Therefore, understanding the mechanisms by which the immune system is modulated by Shh signaling not only advances our knowledge of the control mechanisms of the immune system but also provides insights into the pathogenesis of specific diseases.

Despite the growing interest in Shh in immune regulation, little is known about Shh signaling in immune cells which prompted us to search for the downstream signaling targets of Shh in CD4+ T cells. Here we demonstrate that exogenous Shh can provide co-stimulatory activity, potentiating CD3-mediated CD4+ T cell proliferation and cytokine production in a dose-dependent manner. cDNA microarray analysis reveals that some common downstream targets are shared by Shh and CD28 signaling pathways.

**Methods**

**Mice and antibodies**

C57BL/6N mice were obtained from Charles River Laboratory, USA, and breeding stocks were maintained in the specific-pathogen-free area of Laboratory Animal Unit, University of Hong Kong. Female mice that were 6- to 10-weeks old were used in this study. All experiments were performed in accordance with the Animal (Control of Experiments) Ordinance of Hong Kong SAR. Purified monoclonal anti-CD3ε, anti-CD28, biotinylated anti-CD8α, biotinylated anti-CD4, PE-conjugated anti-CD4, FITC-conjugated anti-IFN-γ, FITC-conjugated anti-tumor necrosis factor (TNF)-α, isotype control antibodies and streptavidin-conjugated CyChrome were obtained from BD PharMingen, CA, USA. Polyclonal goat anti-Shh antibody, normal goat IgG control antibody and rabbit anti-goat IgG–FITC antibody were obtained from Santa Cruz Biotech., CA, USA. The neutralizing anti-Shh antibody 5E1 (Development Studies Hybridoma Bank, IA, USA) was purified from hybridoma supernatant using protein G columns.

**T lymphocytes isolation and immunostaining**

CD4+ or CD8+ T cells were isolated by MACS magnetic beads depletion using the Pan-T cell Isolation Kit (Miltenyi Biotec, Germany) with a slight modification of the manufacturer’s protocol. Briefly, single-cell splenocyte suspension was first incubated with biotinylated antibody cocktail provided by the kit and additional biotinylated anti-CD8α or anti-CD4 antibody was added at 3 μg per 10⁶ cells. After incubation at 4°C for 15 min, the cells were washed, further incubated with antibiotin magnetic beads at 4°C for 15 min and followed by separation on a MACS LS separation column (Miltenyi Biotec). Eluted CD4+ T or CD8+ T cells consistently attained a purity of 90-95%. For immunostaining, after removal of RBCs, total spleen cells were first incubated with biotinylated anti-CD8, PE-conjugated anti-CD4 and goat anti-Shh antibodies mix, followed by staining with rabbit anti-goat IgG–FITC and streptavidin-conjugated CyChrome mix. Cells were harvested and analyzed using FACSCalibur with CellQuest software (BD Biosciences, CA, USA).

**T cell stimulation and proliferation assays**

Purified CD4+ T cells were labeled with 10 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes Inc., OR, USA) at room temperature for 10 min. After extensive washing, 10⁵ cells were stimulated for 3 days in 96-well flat-bottomed plates pre-coated with 1 μg ml⁻¹ anti-CD3ε antibody, with or without exogenous Shh, 5E1, anti-CD28 or mouse IgG1 isotype antibody, followed by analysis on FACS Calibur with CellQuest software. Recombinant mouse Shh, amino-terminal peptide with endotoxin level <0.01% was obtained from R&D Systems, Inc., MN, USA. In separate experiments, CD4+ T cells were stimulated as described above and the total number of viable cells in each well were determined on day 3 using trypan blue dye exclusion method. The percentage of proliferating cells was also determined on day 3 by pulsing with 10 μM 5-bromo-2-deoxyuridine (BrdU) for 6 h before staining with the BrdU Flow Kit (BD PharMingen). [³H]thymidine ([³H]Tdr) incorporation assays were set up as above in 96-well flat-bottomed plates precoated with various amount of anti-CD3 and anti-CD28 antibodies with or without Shh-N as indicated in individual experiment and on day 3 were pulsed with 1μCi [³H]Tdr for a further 12–16 h. Cells were harvested on glass fiber filters and proliferation was determined by scintillation counting.

**Cytokine measurement using cytometric bead arrays and intracellular staining**

Purified CD4+ T cells were stimulated as described in the [³H]Tdr incorporation assay and supernatants were harvested 3 days after culture and stored at –20°C until use. Cytokine levels were measured using mouse Tn1/Tn2 Cytokine Bead Array (BD Biosciences) according to manufacturer’s instructions. The samples were harvested using FACSCalibur and were analyzed using CBA software (BD Biosciences). In separate experiments, CD4+ T cells were stimulated for 24 h, followed by intracellular staining for IFN-γ and TNF-α using the Cytofix and Cytoperm intracellular staining system (BD PharMingen). Cells were then analyzed using FACSCalibur with CellQuest software.

**Conventional and quantitative reverse transcription–PCR**

Total RNA was purified from cultured cells using RNeasy kits with DNase I digestion (Qiagen GmbH, Germany) according to manufacturer’s instructions. RNA (2 μg) was reversed
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transcribed using 100 ng of random hexamers, 0.5 mM deoxynucleoside triphosphates (dNTPs), 10 mM dithiothreitol (DTT) and 50 U of SuperScript II reverse transcriptase (Invitrogen Corp., CA, USA) in 1× First-Strand Buffer at 42°C for 90 min. Conventional PCR was performed using 1 µM forward and reverse primers, 200 µM dNTPs and 2.5 U Taq polymerase (Invitrogen Corp.) in 1× reaction buffer at 94°C for 30 s, 55°C for 30 s and 72°C for 60 s for 40 cycles. Primers sequences—Ptch: forward 5′-ttggtgcatctggtggc-3′ and reverse 5′-ctgggtctctgatttcg-3′ (product size: 487 bp); Smo: forward 5′-ttggtgcatctggtggc-3′ and reverse 5′-ctggttctctgatttcg-3′ (523 bp) and β-actin: forward 5′-gagagggaaatcgtgcgtgac-3′ and reverse 5′-agctcggtacaagctcgccga-3′ (534 bp). Quantitative (real-time) PCR was performed using 500 nM forward and reverse primers in 1× SYBR Green PCR Master Mix (Applied Biosystems, CA, USA) and amplified for 50 cycles at 94°C for 15 s, 55°C for 15 s and 72°C for 30 s using ABI Prism 7700 Sequence Detector (Applied Biosystems). Primer sequences for quantitative PCR (qPCR)—Btg-2: forward 5′-tggagccgagtctcgcagc-3′ and reverse 5′-ggggtcccagtgtggttgat-3′; IL-2: forward 5′-atgggccagctcaagcagtgtgt-3′ and reverse 5′-gagacacctcaggacacggt-3′ and CIS-1: forward 5′-tggagccgagtctcgcagc-3′ and reverse 5′-taggcagcaccgagttcagtgcac-3′; cyclin A2: forward 5′-acctggtctctgatttcg-3′ and reverse 5′-tggtgggtctcctgatttcg-3′; Ptch: forward 5′-atgggccagctcaagcagtgtgt-3′ and reverse 5′-gagacacctcaggacacggt-3′ and Bcl-xL: forward 5′-tgaggctgtggtctcgggtg-3′ and reverse 5′-aagacccacccagcagcagc-3′; IL-2: forward 5′-gacccagctcaagcagtgtgt-3′ and reverse 5′-tacctgaatggcctccaa-3′ and CIS-1: forward 5′-ctggttctctgatttcg-3′ and reverse 5′-taggcagcaccgagt-3′. Signal of a gene (x) was normalized with β-actin (r) using the formula δCt = Ct(x) − Ct(r). The differential expression signal was calculated as dCt = δCt (Shh or anti-CD28) − δCt (anti-CD3 alone) and expressed as relative fold of change using the formula 2−δCt.

cDNA microarray experimental design

CD4+ T cells were purified from C57BL/6N mouse spleens by MACS and subjected to stimulation with plate-bound anti-CD3 antibody (coated at 1 µg ml−1) for 12 and 24 h. Stimulation with anti-CD3 antibody alone was used as the reference group. For the treatment group, exogenous Shh-N or anti-CD28 antibody was added to the anti-CD3 stimulation culture at a final concentration of 2 µg ml−1 and cultured for the same period of time.

Probe labeling and hybridization

Total RNA was extracted using TRIzol reagent (Invitrogen Corp.) according to manufacturer’s instruction. Detailed protocols for probe labeling and microarray hybridization are available from the TIGR Web site (http://www.tigr.org/tdb/ microarray). Briefly, 10 µg of DNA treated total RNA was reverse transcribed using 6 µg random hexanucleotides primer, 0.5 mM deoxyadenosine triphosphate, 0.5 mM deoxyctydine triphosphate, 0.5 mM deoxyguanosine triphosphate and 0.5 µM deoxythymidine triphosphate 0.2 mM aminoallyl-deoxyuridine triphosphate, 10 mM DTT and 400 U of SuperScript reverse transcriptase II (Invitrogen Corp.) in 1× First-Strand Buffer at 42°C for 3 h. After hydrolyzing the RNA with 200 mM NaOH and 100 mM EDTA at 65°C for 15 min, the un-incorporated dNTPs and free amines were removed by spinning and washing with distilled water through a Microcon YM-30 column (Millipore, MA, USA). cDNA was lyophilized, re-suspended in 0.1 M Na2CO3, pH 9.0 and reacted with N-hydroxysuccinimide-ester Cy3 or Cy5 dye at room temperature for 1 h. Sodium acetate, pH 5.2, was added to the reaction mix at a final concentration of 100 mM and the un-coupled dyes were removed using Qiagen PCR Purification Kit. Cy3- and Cy5-labeled cDNA probes were combined, lyophilized and re-suspended in 25 µl hybridization buffer containing 50% formamide, 5× standard saline citrate (SSC), 0.1% SDS, 20 µg mouse COT1 DNA and 20 µg poly(A)-DNA. Microarray slides were pre-hybridized with buffer containing 5× SSC, 0.1% SDS and 1% BSA at 42°C for 1 h, and then washed with distilled water, ethanol and were dried. Heat-denatured probe was applied to the slide and covered with a 22 × 60-mm cover slip. Microarray slide was secured inside a hybridization chamber (Corning Inc., USA) and incubated in a water bath at 42°C for 18 h. Hybridized slides were washed for 4 min at 42°C in 1× SSC, 0.2% SDS, and then 2.5 min at room temperature in 0.1× SSC, 0.2% SDS and 2.5 min at room temperature in 0.1× SSC, followed by a brief wash in distilled water. The glass slides were immediately dried and subjected to scanning using the ScanArray 5000 (Packard BioChip Technologies, Inc.). The images were stored in TIFF format and were analyzed using GenePix Pro 4.0 software (Axon Instruments, Foster City, CA, USA).

Microarray data normalization and analysis

Three independent experiments were carried out for each condition and the dyes were flipped in one of the three experiments. The fluorescence signal of each spot from the entire data set was normalized using total intensity normalization (23). Signal derived from anti-CD3 antibody stimulation alone was used as the reference and a ratio of median fluorescence intensity was generated such that a positive value represented up-regulation of gene expression induced by Shh or anti-CD28 antibody treatment while a negative value represented down-regulation of gene expression induction. Fluorescence intensity was generated such that a positive signal from the Shh or anti-CD28 stimulated sample was reflected a down-regulation of such gene in these treatments. Low intensity usually resulted in skewing the ratio value reflected a down-regulation of gene expression induced by Shh or anti-CD28 antibody treatment while a negative value represented up-regulation of gene expression induced by Shh or anti-CD28 antibody treatment while a negative value represented down-regulation of gene expression induction. Low intensity usually resulted in skewing the ratio. Statistical significance was calculated using Student’s t-test.

Results

Shh receptors expression in resting and activated T lymphocytes

Before determining the effect of exogenous Shh-N peptide on T cell functions, we first characterized the expression pattern of the Shh receptors, Ptch and Smo, in T cells using reverse transcription (RT)–PCR. Resting T cell RNA was prepared from CD4+ and CD8+ T cells purified from un-immunized mice. Activated T cells were prepared by stimulating resting T cells with plate-bound anti-CD3 and anti-CD28 antibodies for 48 h. Total RNA from mouse embryonic limb buds was used as a positive control for the RT-PCR. As shown in Fig. 1(A),

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Exogenous Shh-N amplifies TCR-mediated CD4+ T cell functions

Since Shh receptors were present on T cells, the effect of exogenous Shh-N on T cell functions was examined. Purified CD4+ T cells were labeled with CFSE and subjected to various forms of stimulations. Exogenous Shh-N alone failed to stimulate CD4+ T cells proliferation as indicated by a single peak of CFSE-labeled population after 3 days culture (Fig. 2A). T cell activation markers such as CD69 and CD25 were not up-regulated (data not shown) and the total cell number dropped from \(10^5\) to \(0.30 \pm 0.03 \times 10^5\) in 3 days, providing further evidence that Shh alone did not activate T cells. In contrast, exogenous Shh-N enhanced anti-CD3 antibody-mediated T cell proliferation as there were significantly more cells that underwent cell divisions when compared with anti-CD3 stimulation alone (Fig. 2A and B). Similarly, there were significantly more cells taking up BrdU in the anti-CD3 plus Shh-N treatment group when compared with anti-CD3 alone control (67.72 \pm 5.53% versus 49.86 \pm 1.99%, \(P < 0.05\), Fig. 2C). Correspondingly, in a 3-day stimulation of anti-CD3, the total cell number increased from \(10^5\) to \(2.62 \pm 0.09 \times 10^5\) in the presence of Shh-N and it was significantly higher than that without the peptide (2.17 \pm 0.02 \times 10^5, \(P < 0.01\), Fig. 2D). The Shh-N used was a recombinant peptide of 180 amino acid residues. Instead of using an irrelevant peptide of similar size with random amino acid residues that could be difficult to predict its non-specific effect on the cells, we used the Shh-neutralizing antibody 5E1 to confirm the specificity. The enhancement of T cell proliferation was Shh specific because it was blocked completely by the addition of the Shh-neutralizing antibody 5E1, while the control mouse IgG1 antibody had no effect (Fig. 2A–D). However, when compared with soluble anti-CD28 antibody, the co-stimulatory effect of Shh-N was less potent (Fig. 2A–D). To further delineate the effect of Shh on CD4+ T cell proliferation, we measured \(^{3}H\)Thymidine incorporation. The level of TCR stimulation was titrated using various combinations of anti-CD3 and anti-CD28 antibodies while a fixed concentration of 2 \(\mu\)g ml\(^{-1}\) exogenous Shh-N was added to the culture. As shown in Fig. 3(A), the co-stimulatory effect of Shh-N on CD4+ T cell proliferation was most prominent at sub-optimal level of TCR stimulation. Four days after the initial culture, there was approximately a 7- to 9-fold increase in thymidine uptake at low TCR stimulation level (1 \(\mu\)g ml\(^{-1}\) anti-CD3 with or without 2 \(\mu\)g ml\(^{-1}\) anti-CD28) while the induction was only \sim 40-50% at a high TCR stimulation level (5 \(\mu\)g ml\(^{-1}\) of anti-CD3 with or without 5 \(\mu\)g ml\(^{-1}\) anti-CD28). Moreover, at the sub-optimal TCR stimulation level using 1 \(\mu\)g ml\(^{-1}\) plate-bound anti-CD3 antibody, a dose-dependent enhancement effect of Shh-N on CD4+ T cell proliferation was observed (Fig. 3B).

In addition to the proliferative response, the effect of Shh on cytokine production was examined at different TCR stimulation levels and in the presence of different doses of Shh. Shh-N treatment alone did not stimulate CD4+ T cells to produce any detectable amount of cytokines (data not shown). In concordance with the proliferative response, at sub-optimal TCR stimulation, Shh also enhanced the production of IL-2, TNF-\(\alpha\), IFN-\(\gamma\) and IL-4 in a dose-dependent manner (Fig. 4A). Notably, the enhancement effect of Shh on different cytokines...
varied with the strength of TCR stimulation. At weaker TCR stimulation strength (1 μg ml⁻¹ anti-CD3 with or without 2 μg ml⁻¹ anti-CD28), the enhancement of IFN-γ, a Th1 cytokine, was ~3- to 4-fold (P < 0.01) while IL-4 induction was minimal (<50 pg ml⁻¹, Fig. 4B). With increasing TCR stimulation strength, more IFN-γ was produced but the enhancement effect of the Shh became less significant. On the other hand, the enhancement of the Th2 cytokine IL-4 was more prominent and significant (P < 0.05) at a stronger TCR stimulation level (5 μg ml⁻¹ anti-CD3 and anti-CD28, Fig. 4B). Interestingly, intracellular staining revealed that Shh-N peptide did not significantly increase the number of IFN-γ- and TNF-α-producing

Fig. 2. Exogenous Shh enhances CD3-mediated CD4⁺ T cells proliferation. Purified and CFSE-labeled CD4⁺ T cells were stimulated with plate-bound anti-CD3 antibody (1 μg ml⁻¹) in combination with exogenous Shh (2 μg ml⁻¹), or soluble anti-CD28 (2 μg ml⁻¹), in the presence or absence of Shh-neutralizing antibody 5E1 (20 μg ml⁻¹) or mouse IgG1 isotype control antibody (20 μg ml⁻¹). Flow cytometry was performed 72 h after stimulation and representative histograms were shown in (A). (B) Percent of CFSE-positive cells in each cell division was shown as mean ± SD from three independent experiments. * Represents P < 0.05 when compared with anti-CD3 alone control group. (C) Purified CD4⁺ T cells were stimulated as in (A) and on day 3 were pulsed with 10 μM BrdU for 6 h before staining with anti-BrdU. (D) Purified CD4⁺ T cells (10⁵) were seeded in each 96-well flat-bottomed plate initially and were stimulated as in (A). Total number of live cells in each well was counted on day 3. Data shown in (C) and (D) were mean ± SD from three independent experiments; * and ** represent P < 0.05 and P < 0.01, respectively, when compared with anti-CD3 alone control group.
cells activated by anti-CD3 stimulation at 24 h (Fig. 5) and 48 h (data not shown). This suggests that the increased cytokine production is likely a result of a gradual accumulation over time rather than coming from a sub-population of cells.

**Downstream signaling targets of Shh in CD4+ T cells**

Little is known about Shh signaling in T lymphocytes. In this study, cDNA microarray was used to search for Shh-responsive genes in CD4+ T cells. In order to probe for the early target genes of Shh signaling, mRNA expression was studied at time points 12 and 24 h after stimulation. Fluorescence-labeled (Cy3 or Cy5) cDNA was derived from purified CD4+ T cells stimulated with plate-bound anti-CD3 antibody alone or with exogenous Shh-N, and subjected to hybridization on a single glass slide. Control experiments were carried out in parallel using cDNA derived from CD4+ T cells stimulated with anti-CD3 antibody and anti-CD28 antibodies. In both experiments, stimulation with anti-CD3 antibody alone was used as the reference cDNA. Modulation of gene expression induced by Shh-N treatment was limited and a cut-off of 1.5-fold difference was used. Selected genes that were differentially expressed by the treatment groups compared with anti-CD3 antibody treatment alone at 12 and 24 h after stimulation are presented in Table 1. At T = 24 h, genes related to supporting cell proliferation were up-regulated in both Shh and anti-CD28 treatment and included those for controlling cell cycle (cyclin A), enhancing nucleotide biosynthesis (ribonucleotide reductase M1) and amino acids metabolism (spermidine synthase). Interestingly, cytokine-inducible SH2-containing protein (CIS-1), a cytokine signaling regulator, was also up-regulated. In contrast, B cell translocation gene 2 (Btg-2 or TIS21), which has anti-proliferative effects, was down-regulated in both treatment groups. However, the expression of Bcl-XL, GADD45 and cyclin E were up-regulated by CD28 co-stimulation but not by Shh treatment. Ptcch, cyclin D and cyclin E are the downstream targets of Shh signaling in neuronal cells (24, 25). However, their expressions were not markedly altered by Shh stimulations in activated CD4+ T cells (Table 1).

The expression of selected genes was further validated by qRT-PCR. Since the expression of β-actin was not significantly altered by the addition of Shh-N or soluble anti-CD28 when compared with anti-CD3 stimulation alone (Table 1), it was used as a housekeeping gene for normalization in the qRT-PCR. In concordance with the enhancement in IL-2 production (Fig. 4), IL-2 mRNA expression was significantly up-regulated by both Shh and anti-CD28 treatment 24 h after stimulation (Fig. 6). Furthermore, cyclin A, CIS-1 and Btg-2 were also significantly changed by both Shh and anti-CD28 treatment, confirming the results of the microarray analysis. In addition, the anti-apoptotic factor, Bcl-XL, was up-regulated in CD28 co-stimulation but not following Shh treatment at T = 24 h while Ptcch expression was not significantly changed in either case (Fig. 6). No significant changes in the expression of the tested genes in the Shh treatment group were observed at T = 12 h (data not shown).

**Discussion**

In this study, we have examined several aspects of the contribution of Shh in peripheral T cell immunity. Firstly, the expression of the receptors for Shh ligand on peripheral CD4+ and CD8+ T cells is confirmed. Ptcch and Smo mRNAs are detected on both resting and activated T cells. Upon TCR stimulation, Smo expression is up-regulated in both CD4+ and CD8+ T cells while Ptcch expression is not affected. Ptcch is the binding receptor for Shh ligand. In neuronal cells, Smo mediates Shh signaling through the transcription factor Gli but its activity is suppressed by unbound Ptcch. The up-regulation of Smo may suggest that activated T cells are more sensitive to Shh signaling. However, some studies have indicated that an increase in Smo protein does not necessarily correspond to an increase in Hh signaling but it is the ratio of Hh-bound and unbound Ptcch that determines the cellular response to the Hh protein (26, 27). Hence, the physiological significance of the increase in Smo mRNA transcript in activated T cells needs to be further examined. Apart from Shh receptors, Shh ligand is also readily detectable on the surface of freshly isolated T cells (Fig. 1C), suggesting that Shh signaling is
likely to play a physiological role in either maintaining T cell homeostasis and/or contributing to normal T cell functions. Interestingly, Shh can be produced by follicular dendritic cells (22). This raises the possibility that follicular dendritic cells can mediate a long-range interaction with T cells through the production of a diffusible protein such as Shh.

In addition to Shh receptor expression, we have investigated the effect of Shh signaling on CD4\(^+\) T cell during stimulation. For optimal T cell activation, it is known that secondary or co-stimulatory signals are required in addition to TCR-mediated signaling. Several co-stimulatory pathways have been characterized and include B7/CD28, CD40/CD40L, B7RP/ICOS and OX40L/OX40 (28). Here, we demonstrate that exogenous Shh-N peptide acts in a similar way as other co-stimulatory ligands and potentiates both proliferation and cytokines production by CD4\(^+\) T cells under conditions of sub-optimal TCR stimulation. This extends our previous results that Shh can modulate the response of human T cells (21). We further investigated the molecular basis of Shh signaling in CD4\(^+\) T cells by analyzing gene expression. Compared with co-stimulation provided by anti-CD28 antibody treatment, the magnitude of altered gene expression induced by Shh in anti-CD3 antibody-activated T cells was limited. This was also noted in repeating the microarray analysis using Affymetrix GeneChips (data not shown). It is possible that ligation of anti-CD28 antibody induces a much stronger signal than Shh-N binding to Ptch on T cells as reflected in a greater enhancement of proliferation. In addition, in the experiments reported here, stimulation was delivered by unmodified recombinant Shh-N peptide, which may not be optimal since palmitoylated and cholesterol-modified Shh-N can markedly increase the potency by >30-fold (5). Nevertheless, in concordance with the finding that Shh promotes T cell proliferation in vitro, genes related to DNA replication and
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amino acids biosynthesis (ribonucleotide reductase M1 and spermidine synthase) and cell cycle progression (cyclin A) were up-regulated. Lowrey et al. (20) have previously reported that Shh promotes activated CD4\(^+\) T cells to enter the S/G2 phase of cell cycle while 5E1, the neutralizing anti-

Shh antibody, can cause cell cycle arrest at G1 phase. In primary neuronal precursor cells, Shh signaling promotes proliferation by up-regulating the transcription of cyclin D and cyclin E (29). In addition, Ptch is the target of Shh signaling in neural tube development (24). However, the mRNA expressions of cyclin D, cyclin E and Ptch were not significantly changed by Shh co-stimulation in activated CD4\(^+\) T cells. There have been reports showing that Shh can signal independent of Gli transcription factor (30) and our results suggest that the downstream targets of Shh in T lymphocytes are different from those in neuronal cells.

We demonstrate that Shh regulates a subset of genes in CD4\(^+\) T cells, which are also similarly regulated by CD28 signaling. Microarray analyses have revealed that CD28 signaling predominantly augments TCR/CD3-mediated transcriptional responses (31, 32), leading to the activation of transcription factors such as nuclear factor of activated T cells, nuclear factor-kappaB and AP-1 via protein kinase C-\(\theta\) which results in increased expression of IL-2 and other cytokines (33). CD28 also enhances cell proliferation through modulation of the cell cycle progression machinery (34, 35). Shh co-stimulation in CD4\(^+\) T cells increases IL-2 production, up-regulates CD25 expression and promotes cell cycle progression; hence, it is not surprising that Shh signaling in T cells share some common targets with CD28. As well as increased expression of genes that are general components of the cell proliferation machinery, there is also a synchronized down-regulation of anti-proliferation genes such as the B cell translocation gene 2. CIS-1, a cytokine-induced negative regulator, was also up-regulated in both Shh and anti-CD28 antibody co-stimulation treatment. CD28 co-stimulation also enhances T cell survival. The anti-apoptotic protein Bcl-xL (36) and GADD45\(\beta\), a protein that down-modulates pro-apoptotic Jun N-terminal kinase signaling (37), were both

**Fig. 5.** Shh-N co-stimulation does not increase the number of IFN-\(\gamma\)- and TNF-\(\alpha\)-producing cells. Purified CD4\(^+\) T cells were stimulated with 1 \(\mu\)g ml\(^{-1}\) plate-bound anti-CD3\(\alpha\) antibody with or without 2 \(\mu\)g ml\(^{-1}\) Shh-N peptide for 24 h, followed by intracellular staining for IFN-\(\gamma\) and TNF-\(\alpha\). Representative dot plots were shown and the number in each plot was mean percent positive cells \pm SD from three independent experiments. No statistical significance was found between treatment groups.

### Table 1. Genes differentially regulated by Shh or anti-CD28 treatment in activated CD4\(^+\) T cells

<table>
<thead>
<tr>
<th>Gene</th>
<th>12 h Shh</th>
<th>12 h CD28</th>
<th>24 h Shh</th>
<th>24 h CD28</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclin A</td>
<td>+1.22 (*) ((\pm)0.36)</td>
<td>+1.23 ((\pm)0.31)</td>
<td>+1.53 (*) ((\pm)0.23)</td>
<td>+1.94 (*) ((\pm)0.58)</td>
<td>Control of cell cycle at G1/S and G2/M transitions</td>
</tr>
<tr>
<td>Ribonucleotide reductase M1</td>
<td>+1.07 ((\pm)0.11)</td>
<td>+1.31 ((\pm)0.17)</td>
<td>+1.55 (*) ((\pm)0.05)</td>
<td>+1.96 (*) ((\pm)0.58)</td>
<td>Provide the precursors necessary for DNA synthesis</td>
</tr>
<tr>
<td>Spermidine synthase</td>
<td>+1.39 ((\pm)0.22)</td>
<td>+1.31 ((\pm)0.43)</td>
<td>+1.55 (*) ((\pm)0.27)</td>
<td>+3.55 (*) ((\pm)0.65)</td>
<td>Essential for cell survival and proliferation (amino acids metabolism)</td>
</tr>
<tr>
<td>Cytokine-inducible SH2-containing protein (CIS-1)</td>
<td>+1.03 ((\pm)0.30)</td>
<td>+2.24 (*) ((\pm)0.46)</td>
<td>+1.76 (*) ((\pm)0.82)</td>
<td>+2.81 (*) ((\pm)0.45)</td>
<td>Negative-feedback regulation on cytokine signal transduction</td>
</tr>
<tr>
<td>B cell translocation gene 2 (Btg-2 or TIS21)</td>
<td>-1.09 ((\pm)0.03)</td>
<td>-1.29 ((\pm)0.16)</td>
<td>-1.57 (*) ((\pm)0.08)</td>
<td>-1.91 (*) ((\pm)0.11)</td>
<td>Anti-proliferative protein</td>
</tr>
<tr>
<td>Bcl-XL</td>
<td>-1.19 ((\pm)0.24)</td>
<td>+2.66 (*) ((\pm)0.66)</td>
<td>+1.24 ((\pm)0.12)</td>
<td>+2.13 (*) ((\pm)0.41)</td>
<td>For cell survival</td>
</tr>
<tr>
<td>GADD45(\beta)</td>
<td>-1.12 ((\pm)0.31)</td>
<td>+2.56 (*) ((\pm)0.90)</td>
<td>+1.11 ((\pm)0.10)</td>
<td>+3.37 (*) ((\pm)1.28)</td>
<td>Regulation of growth and apoptosis</td>
</tr>
<tr>
<td>Patched</td>
<td>+1.21 ((\pm)0.21)</td>
<td>+1.20 ((\pm)0.82)</td>
<td>+1.02 ((\pm)0.32)</td>
<td>+1.01 ((\pm)0.37)</td>
<td>Regulate Shh signaling</td>
</tr>
<tr>
<td>Cyclin D</td>
<td>+1.10 ((\pm)0.17)</td>
<td>+1.17 ((\pm)0.24)</td>
<td>+1.12 ((\pm)0.27)</td>
<td>+1.22 ((\pm)0.33)</td>
<td>Regulate cell cycle at G1/S check-point</td>
</tr>
<tr>
<td>Cyclin E</td>
<td>+1.25 ((\pm)0.36)</td>
<td>+1.21 ((\pm)0.77)</td>
<td>+1.20 ((\pm)0.35)</td>
<td>+2.01 (*) ((\pm)0.35)</td>
<td>Regulate cell cycle at G1/S check-point</td>
</tr>
<tr>
<td>(\beta)-Actin</td>
<td>+1.05 ((\pm)0.21)</td>
<td>-1.05 ((\pm)0.15)</td>
<td>+1.03 ((\pm)0.23)</td>
<td>+1.14 ((\pm)0.21)</td>
<td>Cytoskeletal protein</td>
</tr>
</tbody>
</table>

*Numerical values represent the fold of difference in mRNA expression of the indicated gene in microarray analysis. A positive value represents up-regulation and a negative value represents down-regulation. Number shown is the mean (\(\pm\)SD) of three independent experiments.

\(*P < 0.05\) using one-sample t-test for the deviation from mean value of 1 in the control group.
induced by CD28. However, these genes were not modulated by Shh treatment. This is consistent with the finding that exogenous Shh does not enhance the survival of activated T cells (21). Hence, overall the results have indicated that Shh can mediate a co-stimulatory effect in CD4+ T cell activation possibly through a subset of genes that are also regulated by CD28 signaling.

There are several families of proteins that have T cell co-stimulatory properties and these include the CD28 family (CD28/B7 and ICOS/B7R), the TNFR family (CD40/CD40L, OX40L/OX40 and 4-1BB/4-1BBL) and the CD2 family (CD2/CD48, CD58 and CD150) (38–40). Although different co-stimulatory molecules may selectively modulate immune responses, they also share common features. Many of the co-stimulatory molecules identified invariably promote T cell proliferation and cytokine production, but they also have specific roles in shaping immune responses. For instance, ICOS is important for the development of a T,2 response (41) while OX40 promotes the migration of CD4+ T cells to B cell follicles (42). Our data suggest that Shh signaling may serve as another T cell co-stimulatory pathway that is less dominant than CD28. Although exogenous Shh can promote both T,1 and T,2 effector functions, it is still unclear whether in response to quantitative differences under different TCR stimulation Shh may favor the development of a particular functional phenotype of T cells (Fig. 4B). It is also noteworthy that the effect of Shh is examined in total CD4+ T cells in the current study. However, Shh may only act on a subset of CD4+ T cells and further investigation is required to delineate its effect on naive, memory and regulatory T cells separately.

The significance of Shh signaling in T cells is also implicated in pathological conditions. The administration of Shh-neutralizing antibody 5E1 in a rat orthotopic small bowel transplantation model can significantly improve long-term allograft survival with reduced lymphocyte infiltration (P. K. H. Tam, unpublished data). This suggests the in vivo relevance of Shh signaling in a T cell-mediated clinical situation. In fibrotic lungs, Shh expression was up-regulated in the epithelial cells at the site of disease and in the area of tissue remodeling (16). Infiltrating mononuclear cells, alveolar macrophages as well as circulating lymphocytes were also observed to express Ptc. Thus Shh–Ptc signaling through interactions between the damaged parenchyma and infiltrating immune cells may promote tissue repair. In a transgenic mouse model of medullablastoma, Shh and Gli expression was up-regulated in the cerebellum of the diseased mice (15). Furthermore, tumor infiltrating CD4+ and CD8+ T cells expressed high levels of IFN-γ, which induced the expression of Shh in neuronal cells. Hence, the authors proposed a novel role for infiltrating lymphocytes in the tumorigenesis of central nervous system through the regulation of Shh signaling. However, from our findings and those of others demonstrating that Ptc receptor is present on T cells, it is possible that the response of the infiltrating T cells is amplified by the up-regulation of Shh in the tumor, thereby favoring the clearance of tumor cells. The role of T cells in this situation appears paradoxical and further studies are needed to define the precise role of Shh in T cell function under different pathological conditions.

Acknowledgements

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Abbreviations

BrdU 5-bromo-2-deoxyuridine
CFSE carboxyfluorescein diacetate succinimidyl ester
dNTP deoxyribonucleoside triphosphate
DTT dithiothreitol
Hh hedgehog
[3H]TdR [3H]thymidine
Ptc patched
Shh sonic hedgehog
Shh-C sonic hedgehog COOH-terminal domain
Shh-N sonic hedgehog NH2-terminal domain
SSC standard saline citrate
TNF tumor necrosis factor

References

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