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Sonic Hedgehog Promotes Cell Cycle Progression in Activated Peripheral CD4+ T Lymphocytes

Jacqueline A. Lowrey,2*† Gareth A. Stewart,*‡ Susannah Lindey,*‡ Gerard F. Hoyne,*‡ Margaret J. Dallman,§ Sarah E. M. Howie,*§ and Jonathan R. Lamb*‡

Sonic hedgehog (Shh) signaling is important in the growth and differentiation of many cell types and recently has been reported to play a role in T cell development in the thymus. This prompted us to investigate whether or not Shh contributes to the clonal expansion of peripheral CD4+ T cells. In this study, we demonstrate that Shh and other components of the signaling pathway patched, smo, Gli1, Gli2, and Gli3 are expressed in peripheral CD4+ T cells. The addition of the biologically active amino-terminal Shh peptide had no effect on resting CD4+ T cells, but significantly enhanced proliferation of anti-CD3/28 Ab-activated CD4+ T cells. This was not due to antiapoptotic effects, but by promoting entry of T cells into the S-G2 proliferative phase of the cell cycle. Neutralizing anti-Shh Ab reduced T cell proliferation by inhibiting cell transition into the S-G2 phase, suggesting that endogenously produced Shh plays a physiological role in the clonal expansion of T cells. Furthermore, we have observed a significant up-regulation of Shh and Gli1 (glioma-associated oncogene) mRNA in activated CD4+ T cells with or without addition of exogenous Shh, which corresponds with maximal CD4+ T cell proliferation, whereas bcl-2 was only upregulated in activated cells in the presence of Shh. Our findings suggest that endogenously produced Shh may play a role in sustaining normal CD4+ T cell proliferation and exogenously added Shh enhances this response. The Journal of Immunology, 2002, 169: 1869–1875.

Hedgehog (Hh)3 proteins are a highly conserved family of secreted intercellular signaling molecules (1, 2). Originally described in Drosophila as a segment polarity gene (3), several vertebrate homologues have now been discovered, the most extensively characterized being sonic hedgehog (Shh).

Shh is synthesized as a 45-kDa precursor protein, which undergoes autoproteolysis to yield the biologically active amino-terminal domain protein (Shh-N), and a 27-kDa carboxyl-terminal Shh-C protein responsible for the autoprocessing (4, 5). The biologically active Shh-N remains associated with the membrane through cholesterol modification in the autoprocessing step and can exert short-range signaling in this way. There is also evidence for a freely diffusible form of Shh, which would mediate long-range signaling (6–8). Shh signals to neighboring cells via two multitransmembrane proteins patched (ptc) and smoothened (smo). They exist as a receptor complex in which ptc is the ligand-binding subunit and smo is the signaling component. Upon binding of Shh to ptc, an inhibitory effect of ptc on smo is released, allowing smo to transduce the Shh signal across the plasma membrane (9). The signal is then mediated by the Gli family of zinc finger transcription factors, of which three members have now been identified in vertebrates. Gli1 is up-regulated by Shh-secreting cells, while Gli2 and Gli3 appear to be more broadly expressed, suggesting they may also play a role in other pathways (1, 2, 10).

Shh signaling in vertebrates is critical in development, patterning, and cell fate induction in a number of tissues including CNS, limb buds, gut, and lung (1, 11), pituitary gland (12), and pancreas (13). More specifically, it has been reported that Shh induces proliferation in several cell types in vitro such as skin keratinocytes (14), hemopoietic stem cells (15), lung squamous carcinoma cells (16), and neuronal precursor cells (17). Furthermore, mutations in ptc and smo, resulting in constitutive activation of the Shh signaling pathway, are associated with several forms of malignant disease including basal cell carcinoma, medulloblastoma, and rhabdomyosarcoma (18).

Collectively, these results confirm that the Shh pathway is linked to proliferation in many adult cell types, including hemopoietic stem cells (15). Members of the Shh signaling pathway are expressed in the thymus where they appear to function in thymocyte development. They regulate differentiation from the double-negative (CD4+CD8−) to the double-positive (CD4+CD8+) stage of T cell development (19). Shh is also associated with the proliferation of human hemopoietic stem cells and Shh, ptc, and smo transcripts are present in primitive and mature CD19+, CD33+, and CD3+ cell populations (15). Shh has also previously been shown to induce bcl-2 (20), an important regulator of T cell survival (21–24).

Therefore, since the Shh signaling pathway is associated with several aspects of lymphoid cell development, differentiation, and survival, this prompted us to investigate whether or not Shh contributes to the expansion of peripheral CD4+ T cells. In this study, we report the presence of members of the Hh signaling pathway in peripheral CD4+ T cells and secondary lymphoid tissues at both mRNA and protein levels. In addition, we demonstrate that biologically active

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Abbreviations used in this paper: Hh, hedgehog; ptc, patched; smo, smoothened; Shh, sonic Hh; fp, forward primer; rp, reverse primer; ct, cycle threshold; cdk, cyclindependent kinase; MPPF, M phase promoting factor.

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amino-terminal Shh peptide amplifies the proliferation of activated CD4⁺ T cells by enhanced cell entry into the S-G₂ phase of the cell cycle. Neutralizing anti-Shh Ab caused G₁ arrest of the cell cycle. We also report that ligation of the TCR increases transcription of Shh and Gli1 after 72 h of culture. In contrast, in the presence of exogenous Shh, these genes and bcl-2 are up-regulated by 48 h.

Materials and Methods

Mice
C57BL/6J mice were purchased from Harlan Orlac (Bicester, U.K.) and maintained in the Medical Faculty Animal Area Animal Unit at the University of Edinburgh. All experiments were performed in accordance with the animal ethics regulations of the Home Office in the United Kingdom.

Antibodies

Functional grade anti-CD3ε and anti-CD28 Abs were purchased from Insight Biotechnology (Wembley, U.K.). The neutralizing anti-Shh Ab SE1 (Developmental Studies Hybridoma Bank, Iowa City, IA) and the IgG1 isotype control Ab (cell name P3X63Ag8; European Cell Culture Collection, Wiltshire, U.K.) were purified from hybridoma supernatants using protein G columns (Amersham Pharmacia Biotech, Buckinghamshire, U.K.).

Immunocytochemistry

The CD4⁺ T cell proliferation assays (data not shown). Anti-CD4⁺/CD8⁺ Ab (BD Biosciences, Heidelberg, Germany) was used at a dilution of 1/100 for FACS staining. Both anti-Shh N-19 (1/40 dilution) and anti-ptc C-20 (1/60 dilution) Abs for use in immunocytochemistry were goat polyclonal Abs (Abgen, Bioclear, Wiltshire, U.K.). Both anti-Shh and anti-ptc are completely blocked by use of the relevant peptide (data not shown). The secondary Ab for immunocytochemistry was a biotinylated rabbit anti-goat Ab (DAKO, Cambridge, U.K.) used at a dilution of 1/400.

Isolation of CD4⁺ T cells

Single-cell suspensions from pooled C57BL/6 mouse spleens were applied to negative selection CD4⁺ T cell columns (R&D Systems Europe, Abingdon, U.K.) as per the manufacturer’s instructions. Purity was checked using FACS staining with an anti-CD4⁵⁶⁺ Ab and this ranged between 88 and 93%.

Culture of CD4⁺ T cells

CD4⁺T cells were cultured in RPMI 1640 medium (Life Technologies, Paisley, U.K.) supplemented with 10% FCS (Life Technologies), 2 mM g-glutamine, (Sigma-Aldrich, Dorset, U.K.), 20 µg/ml penicillin/streptomycin (Life Technologies), and 50 mM 2-ME (Sigma-Aldrich). Anti-CD4/CD28 Ab activation was conducted at two concentrations, namely, suboptimal (anti-CD3 at 0.25 µg/ml and anti-CD28 at 0.1 µg/ml) and optimal (anti-CD3 at 1 µg/ml and anti-CD28 at 5 µg/ml). Tissue culture plates (Corning Glass, Corning, NY) were coated with the anti-CD3 Ab for 90 min at 37°C and then incubated for 10 min at 25°C, 40 min at 48°C, then 5 min at 95°C.

T cell proliferation assays

The CD4⁺ T cells were cultured as above in 96-well plates with and without the addition of exogenous Shh or SE1. They were pulsed after 48 h of anti-CD3/CD28 activation with 20 µl of [³H]Tdr (50 µCi/ml; Amersham Pharmacia Biotech), harvested at 72 h, and read on a betaplate scintillation counter (Wallac, Milton Keynes, U.K.).

Cell cycle analysis

The CD4⁺ T cells were cultured as above in 48-well plates with and without the addition of exogenous Shh or SE1. At 72 h postactivation, the cells were spun at 13,000 rpm for 7 min, then resuspended in citrate buffer. Cell cycle analysis was conducted using the Vindelov method (25). Briefly, the cells were trypsinized (Sigma-Aldrich) to expose the nucleus before being stained with propidium iodide (Sigma-Aldrich). Cell cycle analysis was then performed on an Epics XL flow cytometer (Beckman Coulter U.K., Buckinghamshire, U.K.). The machine counted 30,000 nuclei in each sample and the software analyzed the percentage of cells in each stage of the cell cycle: sub-G₁, G₁, S, and G₂-M phases. From these values, the percentage of live cells (G₁, S, and G₂-M) was calculated and, from this, the percentage of live cells in the G₁ and S-G₂ phases.

Statistics

A paired t test using a one-tailed p value was used to test the significance of differences in [³H]Tdr incorporation or percentage of CD4⁺ T cells in the proliferative S-G₂ phase with and without the addition of Shh or anti-Shh Ab. Values of p < 0.05 were considered to be significant.

RNA isolation

CD4⁺ T cells were cultured as above in 48-well plates with and without the addition of exogenous Shh or SE1. At various time points (24, 48, 72 h) following activation, the CD4⁺ T cells were spun at 800 for 5 min and then resuspended in lysis buffer provided as part of the RNAeasy kit used for the RNA isolation (Qiagen, Crawley, U.K.). Any contaminating DNA was then digested by treating the RNA with DNase I (Life Technologies) according to the manufacturer’s instructions. To check that no contaminating DNA remained, a PCR was conducted using genomic β-actin primers (forward primer (fp), 5’-CCACAACTGGGACACATG-3’ and reverse primer (rp), 5’-GTCCTCAAAACATGATCTGGTGATC-3’) (MWG Biotec, Ebersberg, Germany). The PCR program was as follows: 35 cycles of 30 s at 94°C, 1 min at 58°C, 2 min at 72°C, followed by a 5-min 72°C extension then 4°C hold. This was conducted on a PTC-200 Peltier thermal cycler (MJ Research, Cambridge, MA).

RT-PCR

Reverse transcription of the RNA was conducted using Moloney murine leukemia virus-reverse transcriptase (all components Promega, Southampton, U.K.). Tubes were incubated at 37°C for 45 min, then at 95°C for 5 min to allow the reverse transcription to take place. The following primer pairs were used for the PCR: Shh fp, AGGGGGGTTTGGGAGAAGAG; Shh rp, GATTCTATAGTGAACCACTG; ptc fp, ATCGATGGGAGATC; ptc rp, CTGGCTTCGTGCTGATTTGCC; smo fp, CTAACACGTCCAACACTG; smo rp, GCCTTGCTTCGTGCTGATTTGCC; flb fp, AGGAGGACCAAGATTG; and flb rp, AACAGATCATGCTCTAGTCTC.

The PCR conditions used were: 35 cycles of 1 min at 94°C, 1 min at 65°C (60°C for Shh and Gli1), 2 min at 72°C followed by an extension of 5 min at 72°C and a 4°C hold.

Real-time PCR

Unless otherwise stated, all materials for real-time PCR were supplied by Applied Biosystems U.K. (Cheshire, U.K.). Four hundred nanograms of RNA was reverse transcribed using the Multiscribe RT kit. Samples were incubated for 10 min at 25°C, 40 min at 48°C, then 5 min at 95°C to allow the reverse transcription to take place. cDNA samples were then diluted 1/5 in nuclease-free water (Promega). The PCR step was conducted using Taqman Universal PCR Mastermix, a primer/probe mix specific to the gene of interest and a primer/probe mix specific to 18S RNA control reagent. The following primer/probe sequences were used: Gli1, 5’-TCTCAACAGTGAAGATCTGGAGATC; Shh, 5’-CTGCGCTAGTCGCTGCAAGACCAAGAATGGCC; TAGTCGTAGTTGAGTGC; and Gli1 rp, GACCCATGGTGGACAGAAAATGG; flb rp, GCAGTCATGTGGGACAGAAAATGG; and flb rp, GCAGTCATGTGGGACAGAAAATGG.

The probes were all labeled with the fluorescent dye FAM. The prim-er/probe mix for 18S was supplied by Applied Biosystems which was labeled with the fluorescent dye vic. Each cDNA sample was run in duplicate using Rotor-Gene 3000, with 18S used as a control. Significant differences in the cycle threshold (ct) values were determined using the comparative ct method (26).

The expression of genes of interest was determined using the comparative ct method (26). The cycle threshold (ct) values of the target gene were compared to the ct values of the control gene 18S (internal standard) using the following formula: ΔΔct = Δct(target) - Δct(18S). The results were expressed as the relative expression level (target/18S) of the transcript of interest. The primer/probe mix for 18S was supplied by Applied Biosystems which was labeled with the fluorescent dye vic. Each cDNA sample was run in duplicate using Rotor-Gene 3000, with 18S used as a control. Significant differences in the cycle threshold (ct) values were determined using the comparative ct method (26). The cycle threshold (ct) values of the target gene were compared to the ct values of the control gene 18S (internal standard) using the following formula: ΔΔct = Δct(target) - Δct(18S). The results were expressed as the relative expression level (target/18S) of the transcript of interest.
each sample, ct is the number of cycles needed to result in a signal crossing a set threshold. Each sample yielded two ct values, one for the gene of interest and one for the 18S housekeeping control. The ct values were then transported to a Microsoft Excel spreadsheet and analyzed to give a value representing the relative mRNA levels present for the gene of interest linearly as per the manufacturer’s instructions.

**Results**

**Expression of Hh signaling pathway components in resting and activated peripheral CD4+ T cells and secondary lymphoid tissue**

Expression of mRNAs encoding Shh, ptc, Smo, and Gli1 was investigated using RT-PCR. RNA from adult thymus was used as a positive control and was compared with the expression of these genes in both resting (t = 0) and anti-CD3/CD28 Ab-activated (t = 72 h) CD4+ T cells. Specific transcripts for Shh, ptc, Smo, and Gli1 were detected in both resting and activated CD4+ T cells (Fig. 1).

To verify the presence of components of the Hh signaling pathway in the peripheral immune system, expression of the Shh and ptc proteins was investigated in the spleen and lymph node using immunocytochemistry. Both ptc- and Shh-expressing cells were present in the spleen (Fig. 1, B–D) and lymph nodes (data not shown).

**Shh peptide promotes peripheral CD4+ T cell proliferation**

Given that the Shh signaling pathway is both present in peripheral CD4+ T cells and has proliferative effects on many cell types, we investigated whether or not Shh modulates peripheral CD4+ T cell proliferation. Purified peripheral CD4+ T cells were cultured with and without the addition of the biologically active amino-terminal Shh peptide. An initial titration curve established that 500 ng/ml was the optimal dose of the Shh peptide to enhance proliferation of peripheral CD4+ T cells (data not shown), and this concentration was used in all subsequent experiments. Shh was added to CD4+ T cells that were resting, maximally stimulated with anti-CD3 (1 μg/ml) and anti-CD28 (5 μg/ml) Abs or suboptimally activated with anti-CD3 (0.25 μg/ml) and anti-CD28 (0.1 μg/ml).

No significant difference in the degree of proliferation was observed following the addition of the Shh peptide in resting CD4+ T cells (Fig. 2A). In maximally stimulated T cells, the Shh peptide was added at two time points, namely, 24 h before (t = −24 h) or at the time of anti-CD3/28 activation (t = 0). However, no significant difference in the level of proliferation as determined by [3H]Tdr incorporation was detected at either time point (Fig. 2A).

Since Shh appeared to have no modulatory effects on CD4+ T cells that had been maximally stimulated with anti-CD3/28 Ab treatment, these experiments were repeated in the presence of suboptimal anti-CD3 (0.25 μg/ml) and anti-CD28 (0.1 μg/ml) stimulation. The Shh peptide was added into culture at t = −24 h and t = 0 relative to activation as before. Fig. 2B shows the increase in [3H]Tdr incorporation with addition of the Shh peptide from which it was calculated that Shh peptide at t = 0 produced a significant increase in CD4+ T cell proliferation ranging from 76.2 to 128% (mean, 101.6%; n = 3; p < 0.01). Addition of Shh peptide 24 h before suboptimal anti-CD3/28 activation also produced a significant increase in proliferation ranging from 16 to 63% (mean, 46%; n = 3; p < 0.04; Fig. 2B).

![FIGURE 1](Image) Expression of components of the Hh signaling pathway in peripheral CD4+ T cells. A, RT-PCR analysis of the expression of Shh (S), Gli1 (G), smo (Sm), and ptc (P) for adult mouse normal thymus (a, as positive control), activated CD4+ T cells (b), and resting CD4+ T cells (c), with size of product given (bp). H2O = negative control (d). B–D, Protein expression of Shh (C) and ptc (D) in the spleen as determined by immunocytochemistry. An isotype control is also shown (B). Original magnification, ×400.

![FIGURE 2](Image) Exogenous Shh increases proliferation of suboptimally activated CD4+ T cells. Proliferation of CD4+ T cells was measured by [3H]Tdr incorporation at 72 h postactivation. Data given are mean cpm counts from three separate experiments in each case. Those graphs showing significantly higher proliferation of CD4+ T cells with addition of Shh are marked with an asterisk. *, p < 0.01; **, p < 0.04. A, Proliferation of resting CD4+ T cells in medium alone (○) or cultured with 500 ng/ml Shh peptide added at t = 0 (●); CD4+ T cells optimally activated with anti-CD3 (1.0 μg/ml) and anti-CD28 (5 μg/ml) Abs alone (□) or with 500 ng/ml Shh added (■) at either time 0 or 24 h before activation. B, Proliferation of CD4+ T cells suboptimally activated with anti-CD3 (0.25 μg/ml) and anti-CD28 (0.1 μg/ml) Abs alone (○) or in the presence of 500 ng/ml Shh peptide (●) added at time 0 or 24 h before activation.
Shh increases proliferation of peripheral CD4⁺ T cells

The effect of Shh on CD4⁺ T cell proliferation was investigated further using cell cycle analysis to allow us to examine whether Shh affected cell survival or promoted entry in the S-G₂ proliferative phase of the cell cycle. As with the [³H]Tdr incorporation studies, this analysis was conducted on resting CD4⁺ T cells and those optimally and suboptimally activated. Exogenous Shh was added at the time of (t = 0) or 24 h before (t = −24 h) anti-CD3/28 Ab activation and the T cells were analyzed 72 h later. In the case of resting CD4⁺ T cells, Shh peptide was added at t = 0, and the cells were analyzed at 24, 48, and 72 h. The percent cells distributed in sub-G₁, G₁, S, and G₂ phases of the cell cycle was analyzed, and from this the percentage live cells in G₁ and S-G₂ phases was calculated. Fig. 3, A and B, shows a representative plot of the cell cycle distribution in the presence or absence of Shh (500 ng/ml) to demonstrate how the cell cycle was analyzed.

The addition of Shh to resting CD4⁺ T cells had minimal effects on cell survival. The percentage of live, nonactivated CD4⁺ T cells was very similar in cultures with and without Shh added (Fig. 3B). The difference in the percentage of cells in the S-G₂ phase was negligible.

\[
\text{Time in culture} \quad \text{Treatment} \quad \% \text{ live cells} \quad \% \text{ live cells in G₁} \quad \% \text{ live cells in S/G₂}
\begin{array}{|c|c|c|c|}
\hline
24 hr & medium & 74.9 & 98 & 2 \\
48 hr & Shh & 78.3 & 97 & 3 \\
72 hr & Shh & 73.1 & 97 & 3 \\
\hline
\end{array}
\]

Anti-Shh Ab inhibits TCR-mediated CD4⁺ T cell proliferation in vitro

Given that exogenous Shh promotes the proliferation of activated CD4⁺ T cells, we were prompted to investigate whether CD4⁺ T cells produce Shh following TCR-mediated signaling. CD4⁺ T cells were activated with anti-CD3/CD28 Abs in the presence of a neutralizing anti-Shh Ab (5E1). The suboptimally activated CD4⁺ T cells were used in this set of experiments because under these conditions the cells showed increased proliferation as determined by both [³H]Tdr incorporation and enhanced entry into the S-G₂ phase of the cell cycle. The addition of anti-Shh Ab at the time of activation resulted in dose-dependent inhibition of proliferation. In the presence of 50 µg/ml anti-Shh Ab, the decrease ranged from 71.3 to 85.1% (mean, 77%; n = 3; p < 0.03; Fig. 4). Inhibition of proliferation was not detected in the presence of the isotype control Ab. These results demonstrate that endogenous Shh is produced by activated CD4⁺ T cells since the neutralizing Ab binds to Shh but not to the receptor, ptc.

Anti-Shh Ab blocks cell entry into S-G₂ phase

The effect of the anti-Shh Ab on the cell cycle was also investigated (Table I). As with the Shh peptide studies, the anti-Shh Ab does not alter the percent live cells in the culture but exerts its effect by blocking the entry of the CD4⁺ T cells into the proliferative S-G₂ phase of the cell cycle. The percent decrease in the proportion of CD4⁺ T cells in S-G₂ with addition of the anti-Shh Ab (50 µg/ml) ranged from 66.2 to 81.6% (mean, 73.4%; n = 3; p < 0.02). This effect was not seen with the isotype control Ab, in which the percentage of CD4⁺ T cells in the S-G₂ phase was very similar (either a slight increase or decrease) compared with medium-only activated CD4⁺ T cells. The percent alteration in the
activated CD4+ T cells were suboptimally activated with anti-CD3 and anti-CD28 Abs and the neutralizing anti-Shh Ab (5E1) or isotype control added at the time of activation. The 5E1 Ab was added at a concentration of either 20 or 50 μg/ml to observe a dose-dependent effect, and the isotype Ab at 20 μg/ml. Proliferation as measured by [3H]TdR incorporation was determined at 72 h postactivation. Representative data from three independent experiments are shown.

Kinetic analysis of expression of Shh, ptc, Gli1, and bcl-2 in activated CD4+ T cells in the presence and absence of exogenous Shh

To analyze the mechanisms of Shh amplification of TCR-mediated activation in CD4+ T cells, the kinetics of expression of components of the Shh signaling pathway and bcl-2 were analyzed in activated CD4+ T cells in the presence and absence of exogenous Shh. CD4+ T cell cultures were set up as before, suboptimally activated with anti-CD3/CD28, and Shh was added at t = 0. RNA was extracted at 24, 48, and 72 h postactivation. It has been reported that a two times or greater increase in the transcription of any gene on at least two occasions is considered to be significant (26, 27). Proliferation assays and cell cycle analyses were also performed concurrently to ensure that the Shh peptide showed enhanced proliferation in these CD4+ T cell cultures.

To perform a time course analysis, the 48- and 72-h samples were normalized to the 24-h RNA sample, assigned a value of 1. In suboptimally anti-CD3/CD28-activated CD4+ T cells in the absence of Shh, we detected a significantly increased transcription of Shh and Gli1, no significant changes were measured for the expression of either ptc or bcl-2 (Fig. 5A). In the presence of exogenous Shh, Shh transcription was increased at both 48 and 72 h. Gli1 transcription increased at 48 h and was maintained at 72 h. Bcl-2 transcripts increased at 48 and at 72 h. However, although ptc transcription was marginally higher at 72 h it did not reach significance (Fig. 5B).

To examine the effect of the Shh peptide on transcription of the various genes, the RNA samples from activated CD4+ T cell cultures with addition of the Shh peptide were normalized against the medium-only activated cultures at equivalent time points (24, 48, and 72 h). No difference in the level of transcription of ptc or Gli1 was seen between activated CD4+ T cells with and without exogenous Shh peptide throughout the time course. Transcription of Shh was significantly reduced at 24 h in activated CD4+ T cells with exogenous Shh peptide added compared with medium-only activated CD4+ T cells (Fig. 6A). Transcription of bcl-2 was significantly increased at 72 h in activated CD4+ T cells with exogenous Shh peptide added compared with medium-only activated CD4+ T cells (Fig. 6B).

Discussion

In this study, we report that peripheral CD4+ T cells express components of the Shh signaling pathway and that activation of this pathway by Shh enhances TCR-induced proliferation by promoting cell entry into the S-G2 phase of the cell cycle.

Shh has a proliferative effects on a variety of cell types including hemopoietic stem cells (14–17, 19). We have reported that Shh and ptc protein are expressed in peripheral lymphoid tissue by immunocytochemistry and that the Shh signaling pathway components Shh, ptc, smo, and Gli1 are present in both resting and activated peripheral CD4+ T cells by RT-PCR. It has been demonstrated that members of the Shh signaling pathway are expressed in the thymus (19). Shh was present on the thymic epithelial cells but not thymocytes, whereas, the receptors, smo and ptc were detected on thymocytes at various stages of development (19). Furthermore, transcripts for Shh, ptc, and smo have been detected in mature CD3+ T cell populations (15), which is in agreement with the findings reported here.

We observed that the addition of exogenous Shh increased CD4+ T cell proliferation significantly in response to suboptimal stimulation with anti-CD3/28 Abs. This enhancement of proliferation was also noted following the addition of Shh 24 h before activation. In contrast, exogenous Shh failed to amplify T cell proliferation induced by optimal doses of anti-CD3/CD28 Abs. It is unlikely that in vivo Ag would be encountered in an environment that would result in the level of activation mediated by saturating doses of anti-CD3 and anti-CD28 Abs in vitro. Thus, Shh may function as a cofactor and contribute to clonal expansion of T cells under physiological conditions of stimulation.

These studies were extended to cell cycle analysis, and, with this more sensitive technique, we observed that exogenous Shh increased proliferation in activated CD4+ T cells if the Shh peptide

Table I. Neutralising anti-Shh Ab inhibits CD4+ T cell entry into the S-G2 phase

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Live Cells</th>
<th>% Live Cells in G1</th>
<th>% Live Cells in S-G2</th>
<th>% Alteration in Cells in S-G2 with +5E1</th>
</tr>
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<tbody>
<tr>
<td>Medium</td>
<td>50.94</td>
<td>78.3</td>
<td>21.7</td>
<td>NA</td>
</tr>
<tr>
<td>5E1, 20 μg/ml</td>
<td>50.12</td>
<td>86</td>
<td>14</td>
<td>35.5</td>
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<tr>
<td>5E1, 50 μg/ml</td>
<td>47.91</td>
<td>96</td>
<td>4</td>
<td>81.6</td>
</tr>
<tr>
<td>Isotype control, 20 μg/ml</td>
<td>54</td>
<td>82.4</td>
<td>17.6</td>
<td>19</td>
</tr>
</tbody>
</table>

*Methodology: CD4+ T cells were suboptimally activated with anti-CD3 (0.25 μg/ml) and anti-CD28 (0.1 μg/ml) antibodies in the absence or presence of neutralising anti-Shh antibody (5E1) added at t = 0 and at a concentration of either 20 μg/ml or 50 μg/ml to show a dose-dependent effect. The isotype antibody was added at t = 0 and a concentration of 20 μg/ml. Cell cycle analyses were carried out at 72 h post-activation. Representative results of three independent experiments are shown.
was added either 24 h before or at the time of activation. Shh appears to promote CD4+ T cell entry into the proliferative S-G2 phase of the cell cycle. The exception to this was the increase in the percentage of live CD4+ T cells observed with addition of exogenous Shh 24 h before suboptimal anti-CD3/28 Ab activation. However, a significantly higher percentage of those live cells entered the proliferative S-G2 phase of the cell cycle with the addition of Shh. This effect of Shh has been reported for several other cell types (14, 17). For example, it has been demonstrated that Shh induced a disproportionate number of keratinocytes in the S-G2 phase of the cell cycle (14). Kenney and Rowitch (17) found that Shh increased the number of neuronal precursor cells in S phase. They also noted that Shh was unable to recruit quiescent cells into the cell cycle and could only sustain cell cycle progression. This may also be true for CD4+ T cells since we found that Shh has no effect on resting, nonactivated CD4+ T cells. Shh increased cell cycle progression only in cells that had been activated and which would have already entered the G1 phase of the cell cycle. The increased proliferation induced by Shh is not reflected in decreased levels of apoptosis as shown by cell cycle analysis. However, addition of Shh peptide did significantly increase the transcription of bcl-2. Shh has previously been shown to induce expression of bcl-2 (20). Bcl-2 is known to play an important role in the regulation of postthymic T cell survival (21–24) and Shh may act, at least in part, by promoting survival of activated cells through the induction of bcl-2.

TCR-mediated signaling in CD4+ T cells can be blocked with anti-Shh-neutralizing Ab; therefore, this would suggest that Shh may be a normal component of the proliferative response. The proposed mechanism by which Shh exerts its proliferative effect was supported by the antiproliferative effect of the anti-Shh Ab. Again, no change was seen in the level of CD4+ T cell death, but the anti-Shh Ab executed its antiproliferative effect by blocking CD4+ T cell entry into the S-G2 phase of the cell cycle with the majority of cells arresting at G1 phase. Since this Ab binds to Shh, but not to its receptor ptc, this indicates that the Ab is blocking effects of endogenous Shh present in the CD4+ T cell culture. Therefore, it would appear that endogenous Shh maintains proliferation perhaps in an autocrine fashion, since blocking of this endogenous Shh results in decreased proliferation. Exogenous Shh serves to enhance this proliferation. This is supported by the quantitation of the Shh signaling pathway components by real-time PCR. Transcription of Shh mRNA was maximal at 72 h, corresponding to maximal proliferation in activated CD4+ T cells both

FIGURE 6. Relative level of Shh and bcl-2 gene expression in activated CD4+ T cells with and without exogenous Shh. A and B, CD4+ T cells were activated with suboptimal concentrations of anti-CD3 and anti-CD28 in medium alone (A) or with exogenous Shh added at the time of activation (B). Cells were collected at 24, 48, and 72 h and RNA was isolated for the measurement of transcripts by real-time PCR. To examine the effect of exogenous Shh peptide on the transcription of Shh (A) and bcl-2 (B), the RNA samples from activated CD4+ T cell cultures with addition of Shh peptide were normalized against the medium-only activated cultures at equivalent time points.
with and without Shh peptide added to the culture. Therefore, it appears that even in normal activation conditions, Shh may function to promote DNA replication by up-regulation of cyclins D and E, thought to be via synthesis of unknown protein intermediates (28–32). It has previously been shown that Shh expression is associated with increased activity of cdk2 and cdk4, important in G1-S transition, in keratinocytes under normal growth conditions (14). It has also been shown that Shh promotes cell cycle progression in proliferating neuronal precursors by maintaining expression of G1 phase cyclins such as cyclin D1, D2, and E, thought to be via synthesis of unknown protein intermediates (17). Recently, Hh signaling in Drosophila has been shown to promote DNA replication by up-regulation of cyclins D and E (33). These mechanisms may account for the ability of Shh to promote CD4+ T cell entry into the S-G2 phase of the cell cycle. An alternative mode by which Shh can promote cell cycle progression, namely, entry into mitosis requires the activation and nuclear translocation of the M phase promoting factor (MPF) (34, 35). The MPF consists of two proteins, cdc2 and cyclin B1. ptc1 can interact with cyclin B1 and prevent nuclear translocation of the MPF and thereby prevent cell cycle progression. With addition of Shh to bind ptc, the release of cyclin B1 is facilitated, and nuclear import of the MPF and subsequently cell cycle progression can take place (36). However, we observed that the effects of Shh on the cell cycle in CD4+ T cells occurred in S phase, which implies that repression of MPF (which controls the latter G2-M phase) cannot fully account for our findings. The repressive effect of ptc on cell cycle progression could perhaps explain why the transcription of ptc mRNA does not increase throughout the course of proliferation as in the case of Shh mRNA and Gli1 mRNA. In summary, we have presented evidence for a link between Shh signaling and sustained and enhanced peripheral CD4+ T cell proliferation. This appears to via promotion of CD4+ T cells into S-G2 phase of the cell cycle. Furthermore, Shh can be produced in an autocrine fashion by the CD4+ T cells themselves, functioning to amplify and maintain clonal expansion.

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References