Sonic Hedgehog Signaling Modulates Activation of and Cytokine Production by Human Peripheral CD4+ T Cells

Gareth A. Stewart, Jacqueline A. Lowrey, Sonia J. Wakelin, Paul M. Fitch, Susannah Lindey, Margaret J. Dallman, Jonathan R. Lamb and Sarah E. M. Howie

*J Immunol* 2002; 169:5451-5457; 
doi: 10.4049/jimmunol.169.10.5451
http://www.jimmunol.org/content/169/10/5451

---

**References**  This article cites **35 articles**, 15 of which you can access for free at:  [http://www.jimmunol.org/content/169/10/5451.full#ref-list-1](http://www.jimmunol.org/content/169/10/5451.full#ref-list-1)

**Subscription**  Information about subscribing to *The Journal of Immunology* is online at:  [http://jimmunol.org/subscription](http://jimmunol.org/subscription)

**Permissions**  Submit copyright permission requests at:  [http://www.aai.org/About/Publications/JI/copyright.html](http://www.aai.org/About/Publications/JI/copyright.html)

**Email Alerts**  Receive free email-alerts when new articles cite this article. Sign up at:  [http://jimmunol.org/alerts](http://jimmunol.org/alerts)
Sonic Hedgehog Signaling Modulates Activation of and Cytokine Production by Human Peripheral CD4⁺ T Cells

Gareth A. Stewart,*† Jacqueline A. Lowrey,*† Sonia J. Wakelin,*‡ Paul M. Fitch,*‡ Susannah Lindey,*† Margaret J. Dallman,§ Jonathan R. Lamb,*† and Sarah E. M. Howie*‡

Sonic hedgehog (Shh) is important in the growth and differentiation of a variety of cell types, including the development of T cells in the thymus. This prompted us to investigate whether Shh signaling is a functional component of the physiological response of human mature CD4⁺ T cells following Ag recognition. In this study, we demonstrate that Shh and its receptor Patched (Ptc) are expressed on resting and activated human peripheral CD4⁺ T cells. In approximately one-half of the randomly selected, anonymous blood donors tested, exposure of anti-CD3/28 Ab-activated CD4⁺ T cells to the biologically active N-terminal Shh peptide increased the transcription of ptc, thereby demonstrating that Shh signaling had occurred. Furthermore, the addition of exogenous Shh amplified the production of IL-2, IFN-γ, and IL-10 by activated CD4⁺ T cells. The synthesis of IL-2 and IFN-γ, but not IL-10, by CD4⁺ T cells was down-regulated by the addition of neutralizing anti-Shh Ab. Cell surface expression of CD25 and CD69 on activated T cells was up-regulated by exogenous Shh, whereas in the presence of the neutralizing anti-Shh Ab expression it was reduced. Collectively, our findings demonstrate that Shh-mediated signaling is a physiological component of T cell responses, which acts to modulate CD4⁺ T cell effector function. The Journal of Immunology, 2002, 169: 5451–5457.

Hedgehog proteins are highly conserved intercellular signaling molecules that function as morphogens in the development, patterning, and cell fate induction in a range of tissues, such as the CNS, limbs, gastrointestinal tract, and lung (e.g., see Refs. 1–4). Originally described in Drosophila as a polarity gene (5), three homologs of these proteins have now been identified in vertebrates of which the biological activity of sonic hedgehog (Shh)³ has been investigated most extensively (1, 2). Shh is synthesized as a precursor protein that is autoproteolyzed to generate an N-terminal domain (Shh-N) in which the biological activity resides and a C-terminal protein that mediates the autoprocessing (6, 7). The biologically active Shh-N domain, through palmitic acid and cholesterol at its N and C termini, respectively, can remain membrane associated and function as a short-range signaling molecule interacting with neighboring cells. Furthermore, as a secreted diffusible molecule, Shh may also deliver long-range signals (8–10). Shh interacts with a receptor complex, which is comprised of two multitransmembrane proteins, Patched (Ptc) and Smoothened (Smo) (11, 12). Ptc is the ligand-binding subunit, which in the absence of Shh inhibits Smo signaling. However, once Shh has bound, Smo is derepressed through a conformational change and transduces the Shh signal across the cell membrane, which is then mediated by the Gli family of zinc finger transcription factors (1–3).

Shh signaling can induce proliferation in a variety of cell types, which include keratinocytes (13), neuronal precursor cells (14), and hemopoietic stem cells (15). Mutations in Ptc and Smo, which result in constitutive activation of this signaling pathway, have been demonstrated in proliferative diseases, such as basal cell carcinoma (16), further illustrating the ability of Shh signaling to induce proliferation. Components of the Shh pathway have now been detected in thymus, in which they are reported to contribute to T cell development (17). However, while the receptors Ptc and Smo are expressed on thymocytes, Shh was found only to be present on thymic epithelial cells. By neutralizing the activity of Shh, it was observed that the differentiation of thymocytes from the double-negative (CD4⁻ CD8⁻) to double-positive (CD4⁺ CD8⁺) stage was increased, whereas the addition of Shh arrested thymocyte development at the double-negative stage (17). It has been reported that Shh can also induce proliferation in human hemopoietic stem cells, and, in addition, the presence of transcripts for shh, ptc, and smo has been demonstrated in mature CD19⁺ and CD3⁺ cell populations (15). However, information on the effects of Shh signaling on the function of peripheral T cells is limited (18).

The contribution of the Shh signaling pathway in the development and differentiation of the immune system prompted us to determine whether the activation of this pathway can influence the human peripheral CD4⁺ T cell repertoire. We report in this study that Shh and Ptc are present on resting and activated peripheral CD4⁺ T cells and that Ptc expression is increased in approximately one-half of the randomly selected, anonymous blood donors tested, by the addition of exogenous Shh, indicating, therefore, that Shh signaling had occurred in the T cells. The addition of Shh enhanced the expression of CD25 and CD69 and cytokine production, namely IL-2, IL-10, and IFN-γ, by CD4⁺ T cells activated by anti-CD3 and anti-CD28 Abs. In the absence of exogenous Shh, the addition of a neutralizing anti-Shh Ab, which recognizes an epitope that overlaps the Ptc binding site of Shh and...
thus blocks signaling by preventing Shh binding (19–23), reduced cell surface expression of CD25 and CD69 on activated T cells. The production of IL-2 and IFN-γ, but not IL-10, was inhibited by the anti-Shh Ab. Taken collectively, our results demonstrate that the induction of Shh signaling in peripheral human CD4+ T cells modulates T cell activation and cytokine production.

Materials and Methods

Isolation of human CD4+ T cells

Human PBMCs were isolated from randomly selected, anonymized, single-donor Buffy coats, obtained from the Blood Transfusion Center (Royal Infirmary of Edinburgh, U.K.), by centrifugal separation over Histopaque 1077 (Sigma-Aldrich, Dorset, U.K.). CD4+ T cells were separated using negative selection affinity columns (R&D Systems, Abingdon, U.K.), according to the manufacturer’s instructions. After separation, the T cells were washed and resuspended in RPMI 1640 culture medium supplemented with 5% heat-inactivated human AB serum, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 2 mM l-glutamine (Life Technologies, Paisley, U.K.). Purity of the CD4+ T cell preparations was analyzed by flow cytometry and was consistently ≥93%.

Immunocytochemistry

Purified CD4+ T cells were spun onto Vectabond-treated microscope slides (Vector Laboratories, Peterborough, U.K.) using a CytoSpin 3 (Shandon Scientific, Runcorn, Cheshire, U.K.), set at 300 rpm for 3 min. The cells were then fixed and blocked with methanol. After blocking endogenous peroxidase in 3% H2O2, sections were loaded onto a Sequenza (Shandon Scientific). Non-specific binding was blocked using normal rabbit serum, and endogenous biotin was blocked using Vector blocking kit (Vector Laboratories), according to the manufacturer’s instructions. Slides were incubated with the primary Ab diluted in appropriate serum for 40–120 min at room temperature, washed with PBS, and incubated with a biotinylated secondary Ab for 30 min. They were washed again, and Vector RTU ABC (Vector Laboratories) was applied and positive signal was detected by the addition of diaminobenzidine (DAKO U.K., Ely, U.K.), with a hematoxylin counterstain. Primary Abs to the N terminus of Shh (N-19, 5 µg/ml, 1/40 dilution; Santa Cruz Biotechnology, Santa Cruz, CA; Insight Biotechnology, Wembley, Middlesex, U.K.) and to the C terminus of Ptc (C-20, 5 µg/ml, 1/40 dilution; Santa Cruz Biotechnology) were used, with a biotinylated anti-goat IgG secondary Ab (DAKO). Both anti-Shh and anti-Ptc Abs were completely blocked by the use of the relevant peptide (data not shown). Images were captured using a ProgRes 3012 camera (Kontron Elektronik, Zürich, Switzerland) through a Zeiss Axioskop microscope (Oberkochen, Germany).

CD4+ T cell cultures

Purified CD4+ T cells (5 × 10^6/ml) were stimulated by the combination of immobilized anti-CD3 (1 µg/ml) and soluble anti-CD28 (5 µg/ml) Abs (BD PharMingen, San Diego, CA). In experiments in which either exogenous soluble N-Shh peptide or anti-Shh Ab and cytokine control was added, this was performed at the initiation of the cultures.

The mouse rShh, a 180-residue N-terminal peptide (R&D Systems), was reconstituted in PBS and added to the cultures. The following primers were used to detect ptc, with a Tm of 58°C, giving a PCR product of 462 bp: forward primer, CCATGTTCCAGTTACCCGAC; reverse primer, CCATCAGCTTCCGTCAAGCACATCGTG.

The demonstration that transcripts for both Shh and Ptc are present in the human lung epithelial cell line A549 served as a positive control.

Real-time RT-PCR

Primers and probes were designed using Primer Express software (PE Biosystems, U.K.). RNA samples for real-time PCR were reverse transcribed into cDNA using TaqMan MultiScribe Reverse Transcriptase kit (PE Biosystems), as per the manufacturer’s instructions. The thermocycler was programmed as follows: 1 cycle at 25°C for 10 min, 1 cycle at 48°C for 2 min at 68°C, followed by a final extension of 7 min at 68°C. This was performed on a PTC-200 Peltier thermal cycler (MJ Research, Massachusetts, MA).

The following primers were used to detect ptc, with a Tm of 60°C, giving a PCR product of 335 bp: forward primer, CAGGGCGTGTAACGTGTGGC; reverse primer, GGCACGGCCGGCCGAACCTCG.

The demonstration that transcripts for both Shh and Ptc are present in the human lung epithelial cell line A549 served as a positive control.

Statistical analysis

A paired t test using a one-tailed p value was used to test the significance of the increase in expression of CD25 and CD69. For comparing data from interest and one for the 18S housekeeping control. The ct values were then analyzed to give a value representing the relative mRNA levels present for each gene of interest and one for the 18S housekeeping control. The ct values were then analyzed to give a value representing the relative mRNA levels present for each gene of interest linearly.

Results

Shh signaling pathway is expressed and functional in human peripheral CD4+ T lymphocytes

To determine the expression of the Ptc and Shh proteins on human peripheral CD4+ T cells, cytokine preparations of purified CD4+ T cells were analyzed using immunocytochemistry. Cell surface expression of both Ptc (Fig. 1A) and Shh (Fig. 1B) was observed. Cells stained with normal goat IgG at the same protein concentration were negative (Fig. 1C). The presence of the Shh signaling pathway in CD4+ T cells was confirmed by RT-PCR because specific mRNAs for both Ptc and Shh were detected in purified
CD4+ T cells and in a control cell line (A549) derived from a human lung carcinoma (Fig. 1D).

It is documented that activation of the Shh pathway results in increased expression of the receptor Ptc. Therefore, to determine whether Shh signaling is active in peripheral CD4+ T cells, the expression of Ptc was analyzed by real-time PCR in both resting (Fig. 1E) and anti-CD3/28 Ab-activated (Fig. 1F) CD4+ T cells in the presence and absence of exogenous Shh. It has previously been reported that a 2-fold increase in gene expression in two or more independent experiments is considered significant (24, 25). The expression of Ptc-specific mRNA induced in resting CD4+ T cells by exogenous Shh is presented relative to the level present in CD4+ T cells cultured in medium alone at 24 h. We observed that the addition of the N-terminal Shh peptide to resting CD4+ T cells over a concentration range of 10–1000 ng/ml failed to induce a significant increase in Ptc transcripts, although 100 ng/ml had a marginal effect (Fig. 1E). In contrast, relative to anti-CD3/CD28 Ab-activated CD4+ T cells that were maintained in medium alone, the addition of the exogenous Shh peptide at concentrations of 10 ng/ml or greater resulted in a marked increase in transcription of ptc (i.e., >2-fold; Fig. 1F).

Shh signaling amplifies CD4+ T cell effector function

To determine whether Shh signaling contributes to CD4+ T cell effector function induced following Ag recognition, T cells were stimulated with anti-CD3/CD28 Abs in the presence of exogenous Shh. We observed that the addition of exogenous Shh added at the initiation of the T cell cultures led to a significant enhancement of anti-CD3/CD28 Ab-induced T cell proliferation (Fig. 2), in a dose-dependent manner (p < 0.05 at 0.01 ng/ml of Shh, and p < 0.001 at 1 ng/ml of Shh), but had no effect on resting CD4+ T cells (data not shown). To further investigate the influence of Shh signaling on T cell function, cytokine production by activated CD4+ T cells exposed to increasing concentrations of exogenous N-Shh peptide was determined. We observed that the addition of Shh (>100 ng/ml) significantly (p < 0.001) enhanced the levels of IL-2 (Fig. 3A), IL-10 (Fig. 3B), and IFN-γ (Fig. 3C). In approximately one-half (6 of 13) of the individuals tested, we noted that the addition of exogenous Shh failed to amplify the proliferation of CD4+ T cells.

FIGURE 1. Shh signaling pathway is expressed and functional in human peripheral CD4+ T lymphocytes. Cell membrane expression of Ptc (A) and Shh (B) on cytopsinsof purified CD4+ T cells detected by immunocytochemistry with hematoxylin counterstain; cells stained with normal goat IgG are shown in C. Ptc and Shh mRNA (D) expression is shown, as measured by RT-PCR in CD4+ T cells (track 2; Ptc; track 6, Shh), and in a positive control cell line (A549) derived from a human lung carcinoma (track 4, Ptc; track 8, Shh); DNA ladder bands are shown in tracks 1, 3, 5, and 7. Expression of Ptc mRNA in resting (E) and anti-CD3/28 Ab-activated (F) CD4+ T cells cultured in the presence of increasing concentrations (10–1000 ng/ml) of N-Shh peptide is shown at 24 h, evaluated by real-time RT-PCR. The real-time reaction is conducted as a multiplex, with 18S RNA as an internal control. Levels of mRNA transcripts are expressed relative to resting (E) and activated CD4+ T cells (F) cultured in the absence of any N-Shh peptide, which are given a value of 1. Representative experiments of n = 5 are presented.

FIGURE 2. Exogenous N-Shh enhances the proliferative response of TCR-activated CD4+ T cells. Purified CD4+ T cells were activated with immobilized anti-CD3 (1 μg/ml) and soluble anti-CD28 (5 μg/ml) Abs in the presence of increasing concentrations (0.01–100 ng/ml) of exogenous N-Shh peptide. Proliferation as determined by [3H]TdR incorporation was measured at 72 h and compared with cultures of anti-CD3/28-activated T cells in the absence of Shh. ***p < 0.001; *, p < 0.05. Representative experiment of n = 6 is presented.
FIGURE 3. Exogenous N-Shh peptide augments the cytokine production by activated human CD4+ T cells. Purified CD4+ T cells were activated with immobilized anti-CD3 (1 μg/ml) and soluble anti-CD28 (5 μg/ml) Abs in the presence and absence of increasing concentrations (10–1000 ng/ml) of exogenous N-Shh peptide. Supernatants were collected at 72 h, and the levels of IL-2 (A), IL-10 (B), and IFN-γ (C) were measured by ELISA and compared with cultures of anti-CD3/28-activated T cells in the absence of Shh. ***p < 0.001. Representative experiment of n = 5 is presented.

In an extension of these studies, the effect of a neutralizing anti-Shh Ab (5E1) on T cell function was investigated. We observed that the addition of the 5E1 at the initiation of the culture period significantly (p < 0.01 for 5 μg/ml of 5E1) inhibited the production of IL-2 (Fig. 4A) and IFN-γ (Fig. 4C) by anti-CD3/28 Ab-treated CD4+ T cells in a dose-dependent manner. In contrast, synthesis of IL-10 was unaffected by the addition of 5E1 (Fig. 4B). In the presence of the isotype control at equivalent concentrations, no inhibitory effects on cytokine production were noted (data not shown).

Modulation of CD25 and CD69 expression on CD4+ T cells by exogenous Shh and neutralizing anti-Shh Ab

To further investigate the effects of Shh on CD4+ T cell activation, the cell surface expression of CD25 and CD69 was analyzed. N-Shh peptide (1000 ng/ml) was added to anti-CD3/28 Ab-activated CD4+ T cells, and cell surface expression of CD25 and CD69 was measured by FACS analysis at 48 and 72 h after treatment. The FACs profiles for the expression of CD25 and CD69 for activated T cells in the presence and absence of Shh (1000 ng/ml) are shown in Fig. 5, A and B, respectively. Optimum up-regulation of CD25 was seen at 72 h of culture, and of CD69 at 48 h of culture. This was further investigated by titrating the dose of Shh used and observing the increase in these activation markers at their optimum time of induction. The increase in expression was dose dependent (Fig. 5C). In the presence of 5E1 (50 μg/ml), the percentage of cells expressing CD25 was reduced, as was the geometric mean fluorescence intensity (Fig. 6A). Similarly, the percentage of T cells expressing CD69 was also increased, as was the geometric mean fluorescence intensity (Fig. 6B).

Discussion

In this study, we have demonstrated that Shh and its receptor Ptc are expressed on human peripheral CD4+ T cells. We also report that the addition of exogenous Shh enhances the effector function of activated T cells, in approximately one-half of the randomly selected individuals tested, confirming that the Shh signaling pathway is active in the peripheral immune system and has proinflammatory activity. Furthermore, the ability of a neutralizing anti-Shh Ab to inhibit proliferation, the expression of activation Ags, and cytokine production following TCR-mediated activation suggest that the induction of Shh signaling is a physiological component of peripheral CD4+ T cell responses.

The recognition of peptide/MHC class II complexes expressed on the cell surface of APCs together with secondary signals, such as ligation of costimulatory receptors, are required for the activation, the expansion, and the induction of effector function of CD4+ T cells (e.g., reviewed in Ref. 26). The results of this study indicate that one action of Shh on the peripheral immune system is to potentiate TCR and costimulatory receptor-mediated signaling and both amplify clonal expansion and enhance the effector function of CD4+ T cells. We have shown that human peripheral CD4+ T cells express both Shh and Ptc, as determined by immunocytochemistry and RT-PCR. Furthermore, we have noted that both Shh and Ptc are present on human CD8+ T cells (data not shown). Our findings are

FIGURE 4. Neutralizing anti-Shh Ab (5E1) down-regulates cytokine production. Anti-Shh Ab (5E1) was added at increasing concentrations (5–20 μg/ml) at the initiation of cultures of purified CD4+ T cells activated by immobilized anti-CD3 (1 μg/ml) and soluble anti-CD28 (5 μg/ml) Abs. Supernatants were collected at 72 h, and the levels of IL-2 (A), IL-10 (B), and IFN-γ (C) were measured by ELISA and compared with cultures of anti-CD3/28-activated T cells without added 5E1. ***p < 0.01; **p < 0.05. Representative experiment of n = 3 is presented.

Downloaded from by guest on June 9, 2017
in agreement with and extend those of Bhardwaj et al. (15), who demonstrated using RT-PCR that mature human CD3+ cells express Shh, Ptc, and smo. In contrast to peripheral T cells, it has been reported that thymocyte populations appear to express only the receptors Ptc and smo (17), which implies that Shh neither mediates interactions between thymocyte populations nor influences the differentiation and expansion of thymocytes through its autocrine activity. Shh signaling induces the expression of Ptc (11) and, therefore, the ability of exogenous Shh to up-regulate ptc expression on human CD4+ T cells activated by anti-CD3/CD28 Ab treatment confirms that this pathway is functional in T cells.

It is well documented that Shh has proliferative effects on a wide range of different cell types, including those of the hemopoietic system (15). In this study, we demonstrate that exogenous Shh enhances TCR-mediated proliferation by CD4+ T cells. The failure of Shh to induce proliferation or increase expression of Ptc on resting CD4+ T cells suggests that it acts as a cofactor, which potentiates TCR-mediated signaling and amplifies clonal expansion. The proliferative activity of Shh is, in part, through its effects on cell cycle (e.g., Refs. 13, 14, 27, 28) and, for example, it has been reported that Shh can increase the number of neuronal precursors in S phase (14). In parallel studies on murine CD4+ T cells, we have noted that Shh promotes their entry into the proliferative S/G2 phase of cell cycle (18). Kenney and Rowitch (14) observed that Shh failed to advance quiescent neuronal cells into the cell cycle, which is consistent with our results that Shh has no activity on resting T cells. Furthermore, although it has been reported that Shh can induce bcl-2 (29), we observed that the addition of Shh failed to increase survival in activated T cells (data not shown) and, therefore, it is unlikely that enhancement of T cell proliferation by Shh is brought about through a reduction in apoptosis (30–32).

For some individuals, we observed that the addition of exogenous Shh failed to enhance the proliferation response. Mutations in Ptc and smo, resulting in constitutive activation of Shh signaling, have been reported (33). Thus, polymorphisms that bring about loss or modulation of function are also possible and would provide one explanation for our observations. Furthermore, there is a large body of evidence, which demonstrates that proliferation can be dissociated from other T cell effector functions, such as cytotoxic activity and the production of cytokines (e.g., reviewed in Ref. 33). Therefore, we investigated the ability of Shh to modulate cytokine production by CD4+ T cells activated by anti-CD3/CD28 Ab treatment. The addition of exogenous Shh to activated T cells increased production of the different cytokines measured in this study, namely IL-2, IL-10, and IFN-γ. There was no evidence that Shh had selective effects on the synthesis of these cytokines. This is in contrast to a previous report in which increasing the threshold of TCR-mediated signaling enhanced IFN-γ production, but had no effect on IL-10 production or T cell proliferation (34). Furthermore, we demonstrate that the addition of exogenous Shh to activated T cells results in the up-regulation of cell surface expression of CD69, implying that the T cells had been further activated by Shh signaling. The expression of CD25 on activated T cells was also enhanced by exogenous Shh. These findings taken together with the observation that Shh enhances the production of IL-2, in part, may be an additional mechanism by which Shh is
able to increase the proliferation of activated T cells. It also suggests that Shh signaling does not act by modulating the threshold of TCR signaling (35). We observed that the addition of the neutralizing anti-Shh Ab was able to inhibit the production of IL-2 and IFN-γ, but not IL-10, in contrast to exogenous peptide, which enhanced the synthesis of these cytokines. This suggests that IL-10 production can be regulated by TCR signaling independently of Shh activation. However, one effect of Shh on peripheral T cells appears to be its ability to amplify cytokine production by activated CD4+ T cells in a nonselective manner.

We demonstrate that the addition of a neutralizing anti-Shh Ab to CD4+ T cells activated by anti-CD3/28 Ab treatment inhibits T cell proliferation, IL-2 production, and the expression of the activation Ags CD25 and CD69. These findings imply that induction of Shh signaling is a physiological component of T cell activation following the ligation of TCR and costimulatory receptors. Furthermore, this result suggests that Shh produced by CD4+ T cells themselves in response to TCR-mediated activation functions in an autocrine manner. In the thymus, Shh is expressed on epithelial cells, and Ptc and Smo on the thymocytes (15), suggesting that Shh signaling can also be delivered by APCs. We have also detected Ptc and smo in macrophages (unpublished data). The expression of receptors for Shh on both T cells and APCs would be consistent with the concept that the effector function of both these cell populations can be modulated by Shh signaling. However, the effects of Shh on APC function remain to be determined.

In summary, we show that the Shh signaling is functional in peripheral CD4+ T cells. Both Shh and its receptor Ptc are expressed on T cells, and they are up-regulated when the T cells are activated by TCR and costimulatory receptor ligation. The functional consequences of this are that T cell proliferation is enhanced and cell surface expression of the activation Ags CD25 and CD69 is increased, as is cytokine production. Furthermore, the ability of a neutralizing Ab to directly inhibit the biological activity of activated T cells suggests that Shh signaling is a physiological component of CD4+ T cell responses.

Acknowledgments
We thank June Stewart and Anne Grant for their expert technical assistance.

References

FIGURE 6. Anti-Shh Ab decreases the expression of CD25 and CD69 on activated CD4+ T cells. Purified CD4+ T cells were activated with immobilized anti-CD3 (1 μg/ml) and soluble anti-CD28 (5 μg/ml) Abs in the presence of the anti-Shh Ab (5E1) at 50 μg/ml. Cell surface expression of CD25 (A) and CD69 (B) at 72 h was compared with that on anti-CD3/28-activated T cells in the absence of 5E1. Representative experiment of n = 3 is presented.