Available online at www.sciencedirect.com

SCIENCE DIRECT®

Virology 320 (2004) 313–329

VIROLOGY

www.elsevier.com/locate/yviro

Tip, an Lck-interacting protein of *Herpesvirus saimiri*, causes Fas- and Lck-dependent apoptosis of T lymphocytes

Muneer G. Hasham and Alexander Y. Tsygankov*

Department of Microbiology and Immunology, Temple University School of Medicine, Philadelphia, PA 19140, USA

Received 28 August 2003; returned to author for revision 19 November 2003; accepted 24 November 2003

Abstract

Saimiriine herpesvirus-2 (*Herpesvirus saimiri*) transforms T lymphocytes, including human, to continuous growth in vitro. *H. saimiri*-induced transformation is becoming an important tool of T-cell biology, including studies of HIV replication. Two proteins of *H. saimiri* subgroup C, Tip and StpC, are essential for T-cell transformation. In spite of the important role of these proteins, their biological functions and the molecular mechanisms of their action remain insufficiently understood. To further elucidate the effects of Tip on T cells, we transduced T lymphocytes, using an efficient lentiviral gene transfer system, to express Tip in the absence of other *H. saimiri* proteins. Our results indicate that Tip specifically inhibits IL-2 production by human T lymphocytes. Furthermore, Tip promotes T-cell apoptosis, which appears to be the reason for the observed decrease in IL-2 production. Finally, the apoptotic effect of Tip in T cells is mediated by Fas and requires the presence of active Lck in the cell.

Keywords: Tip; StpC; Lck; Fas; IL-2; Apoptosis; Lentiviral gene transfer; T lymphocytes; *Saimiriine herpesvirus-2 (Herpesvirus saimiri)*

Introduction

Saimiriine herpesvirus-2 (*Herpesvirus saimiri*) causes transformation of T lymphocytes, thus inducing lethal T-lymphoproliferative diseases in susceptible species. The strains of group C of this virus are capable of transforming human T cells in vitro (reviewed in (Broker and Fickenscher, 1999; Damania and Jung, 2001; Fickenscher and Fleckenstein, 2001; Isakov and Biesinger, 2000; Jung et al., 1999; Meinl and Hohlfeld, 2000; Tsygankov and Romano, 1999)). Although this transformation renders T cells capable of proliferating in a T-cell antigen receptor (TCR)/CD3-stimulation-independent manner similar to that of lymphoblastoid T-cell lines, *H. saimiri*-transformed T cells retain their antigen-specific reactivity and growth dependence on IL-2 (Broker et al., 1993; Weber et al., 1993). This property of *H. saimiri*-transformed T cells makes them a unique experimental tool that can be used for immortalization of antigen-specific T-cell clones, minor populations of T lymphocytes, and growth-defective T cells, as well as for the studies of HIV replication in T cells (Cabanillas et al., 2002; Gallego et al., 1997; Henderson et al., 1999; Lacey et al., 1998; Martin-Villa et al., 1998; Meinl et al., 2001; Nakamura et al., 2001; Pecher et al., 2001; Saadawi et al., 1997; Saha et al., 1996, 1997, 1998, 1999; Vella et al., 1997, 1999, 2002; Zheng et al., 2002).

Two *H. saimiri* proteins, Tip and StpC, have been shown to be essential for *H. saimiri*-mediated transformation (Duboise et al., 1998; Medveczky et al., 1993). The role of StpC in this process is consistent with the oncogenic potential of StpC, evident by its ability to transform fibroblasts (Jung et al., 1991). The oncogenic potential of StpC is thought to be dependent on its ability to facilitate activation of NF-κB (Lee et al., 1999; Merlo and Tsygankov, 2001) or Ras (Jung and Desrosiers, 1995).

Unlike StpC, Tip exhibits no transformation potential in fibroblasts (Jung et al., 1991). However, it has been found that Tip binds to Lck, a T cell-specific Src family protein tyrosine kinase (PTK), through the interactions of two sites of Tip currently called LBD1 and LBD2 with the SH3 and

Abbreviations: EGFP, enhanced green fluorescent protein; kDa, kilodalton; PBMC, peripheral blood mononuclear cells; PTK, protein tyrosine kinase; TCR, T-cell receptor (for antigen); VSV G, vesicular stomatitis virus glycoprotein.

* Corresponding author. Department of Microbiology and Immunology, Temple University School of Medicine, 3400 N. Broad Street, Philadelphia, PA 19140. Fax: +1-215-707-5205.

E-mail address: alexander.tsygankov@temple.edu (A.Y. Tsygankov).

0042-6822/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2003.11.032

kinase domains of Lck, respectively (Biesinger et al., 1995; Fickenscher et al., 1997; Hartley et al., 1999, 2000; Jung et al., 1995a; Lund et al., 1996, 1999). This binding results in tyrosine phosphorylation of Tip (Biesinger et al., 1995; Fickenscher et al., 1997; Hartley and Cooper, 2000; Jung et al., 1995a, 1995b; Wiese et al., 1996) and the activation of Lck (Hartley et al., 1999, 2000; Kjellen et al., 2002; Lund et al., 1997a, 1999; Wiese et al., 1996). Tip has also been shown to facilitate activation of several transcription factors, namely, Stat1 and Stat3 (Hartley and Cooper, 2000; Kjellen et al., 2002; Lund et al., 1997b, 1999) and NF-AT (Hartley et al., 2000; Kjellen et al., 2002; Merlo and Tsygankov, 2001). Although it appears possible that activation of Lck or multiple transcription factors may underlie the essential role of Tip in *H. saimiri*-mediated transformation, the contribution of the known effects of Tip to its role in T-cell transformation remains unclear. Furthermore, Tip-induced downregulation of Lck in a Jurkat T-lymphoblastoid cell line expressing Tip in a stable fashion has also been reported (Guo et al., 1997; Jung et al., 1995b). A study by the same research group indicates that Tip in a complex with p80, a cellular protein, may induce degradation of Lck in the lysosome (Park et al., 2002). Although these results have been interpreted as arguing in favor of a latency-inducing role of Tip, similar to that of the LMP2A protein of Epstein–Barr virus (reviewed in (Longnecker and Miller, 1996)), such a role has not been established for Tip during *H. saimiri*-induced transformation. Overall, the biological functions of Tip remain insufficiently understood. One of the factors limiting our understanding of the biological role of Tip is the lack of appropriate experimental systems in which the effects of Tip on normal T lymphocytes can be studied. Indeed, analysis of the effects of Tip in multiple *H. saimiri*-transformed T-cell clones and lines

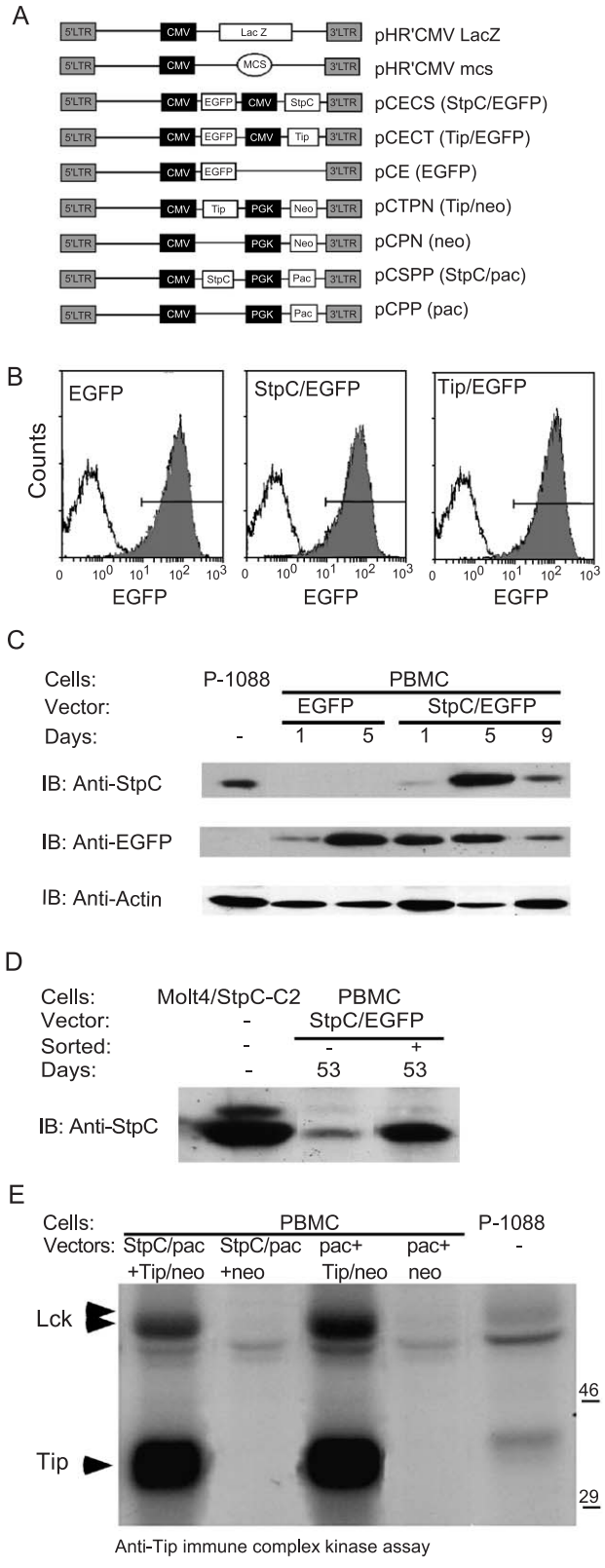

Fig. 1. Transduction and expression of StpC and Tip in T cells. (A) Transfer vectors for StpC and Tip expression. *Lac*Z was excised from pHRCMV-LacZ and replaced with a multiple cloning site (MCS) to generate pHRCMV-MCS. The following fragments were cloned into the MCS of pHRCMV-MCS: EGFP from pLEIN to make pCE; Tip-PGK-Neo, PGK-Neo, StpC-PGK-pac, and PGK-Pac from pMSCV vectors to make pCTPN, pCPN, pCSPP, and pCPP, respectively. CMV-StpC was subsequently cloned into pCE to create pCECS. CMV-Tip was excised from pCEP4/tip and cloned into pCE to create pCECT. (B) Flow cytometry analysis of PBMC transduced with pCE (EGFP alone), pCECS (StpC/EGFP), and pCECT (Tip/EGFP), as indicated in the panels, was conducted 5 days after transduction. Filled and open profiles denote transduced and parental cells, respectively. (C) Immunoblotting of StpC in lysates of *H. saimiri*-transformed T-cell line P-1088 and PBMC transduced with pCE or pCECS as indicated at the top of the panel. EGFP was used as a transduction efficiency control. Actin was used as a loading control. Cells were lysed at various times after transduction as indicated at the top of the panel, and 100 μg of total protein was loaded per lane. (D) Immunoblotting of StpC in the lysate of a stable T-cell line obtained by transducing PBMC with pCECS (50 μg of total protein per lane). T lymphocytes transduced with pCECS were sorted for EGFP green fluorescence on day 5 or left unsorted and lysed on day 53 after transduction. StpC-expressing Molt4 StpC C2 cells were used as a positive control. (E) The anti-Tip immune complex kinase assay of PBMC transduced to express Tip, StpC, or the selection markers neo and pac as indicated at the top of the panel was conducted 4 days after transduction. P-1088 cells were used as positive control for Tip expression. One milligram of total cellular protein was used for each immunoprecipitation sample. The positions of Lck and Tip are indicated by arrowheads. The positions of protein markers and their molecular masses in kilodaltons (kDa) are indicated at the right of the panel. Representative experiments from at least three independent experiments for each panel are shown.

is hindered by the simultaneous expression of StpC and, possibly, other proteins of *H. saimiri* (Fickenscher et al., 1996; Knappe et al., 1997).

We developed a method for the efficient lentivirus-mediated introduction of Tip into primary T lymphocytes to assess the effects of Tip on these cells in the absence of other *H. saimiri* proteins. In this report, we describe the application of this method for the expression of Tip in human T lymphocytes and the analysis of the effects of Tip on T lymphocytes and T-lymphoblastoid cells. Our results have indicated that Tip promotes apoptosis both in primary and lymphoblastoid T cells. Further analysis of this effect has shown that the Tip-induced apoptosis of T cells is mediated by Fas and is dependent on the presence of active Lck.

Results

Efficient transduction of Tip and StpC coding sequences into T cells

While developing the transduction system we pursued the goal of achieving a 100% efficient transfer of Tip or StpC coding sequences into primary T lymphocytes with subsequent high expression levels of both Tip and StpC in the transduced cells. The results of lentiviral transduction of Tip and StpC coding sequences using pCECT and pCECS transfer vectors, respectively, as well as that of the pCE vector used as a marker control (Fig. 1A), demonstrated that under optimized conditions (spin-inoculation in the presence of polybrene at MOI in the range of 3–5), 80–100% of peripheral blood mononuclear cells (PBMCs) were expressing enhanced green fluorescent protein (EGFP) shortly after transduction, indicating highly efficient vector transfer (Fig. 1B). Analysis of various populations of EGFP-expressing PBMCs for cell surface markers indicated that not only T cells, but also B cells, monocytes, and NK cells were successfully transduced (data not shown). Transduction of Jurkat cells was even more efficient than that of PBMC; 70–100% of Jurkat cells expressed EGFP upon treatment with recombinant virus at MOI in the range of 1–3 (data not shown). PBMC and Jurkat cells were also efficiently transduced with the vectors carrying drug selection markers (data not shown).

To further analyze the transfer of Tip and StpC coding sequences into PBMC, their expression in these cells was evaluated as a function of time. The results shown in Fig. 1C indicated that the expression of StpC was evident as early as day 1 after transduction and remained high until day 9, even in unsorted cells. Long-term maintenance of these cells in bulk culture using restimulations with PHA and irradiated feeder cells resulted in a gradual loss of StpC expression. However, sorting of pCECS-transduced PBMC for the EGFP marker at any time after day 5 post-transduction allowed us to obtain T cells expressing EGFP and StpC in a stable fashion (Fig. 1D). Based on flow-cytometric analysis of various PBMC populations, only T cells survived post-transduction cultures for longer than 9 days (data not shown). Therefore, stable EGFP/StpC-positive cells consisted exclusively of T lymphocytes. Similar results were obtained using another StpC-encoding transfer vector, pCSPP, which contained the puromycin resistance gene as a selection marker. PBMC transduced with pCSPP expressed StpC shortly after transduction, and T cells expressing StpC in a stable fashion could be selected by adding puromycin to the culture media (data not shown).

The expression of Tip following transduction of Tip-encoding transfer vectors into human PBMC was determined using immune complex kinase assay. This technique is based on the ability of Tip to co-immunoprecipitate with Lck and to become tyrosine-phosphorylated in these immune complexes. It has been shown previously that Tip expression in most experimental systems cannot be detected by using immunoblotting and that the immune complex kinase assay has to be used instead (Biesinger et al., 1995; Fickenscher et al., 1996, 1997; Wiese et al., 1996). Likewise, in our experiments, Tip could only be detected in T cells using the immune complex kinase assay. The results shown in Fig. 1E indicated that Tip was expressed in PBMC transduced with the pCTPN

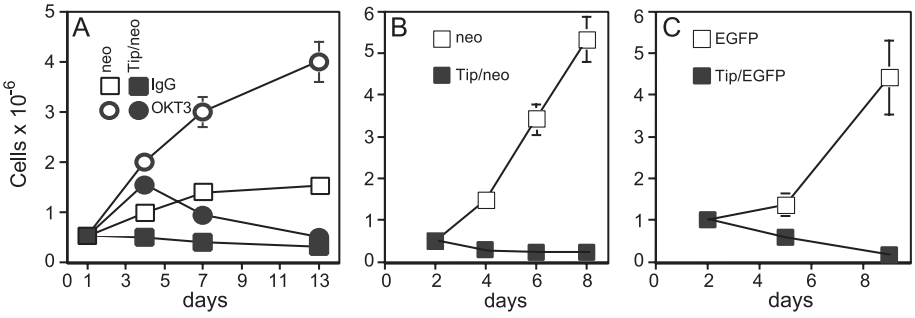

Fig. 2. Suppression of T-cell growth as a result of the expression of Tip. (A) PBMCs transduced with pCTPN or pCPN were activated with the anti-CD3 mAb OKT3 or an isotype control IgG on day 1 after transduction. Jurkat cells were transduced with pCTPN or pCPN (B) or pCECT or pCE (C). The number of viable cells was determined in cultures using trypan blue exclusion at several time points. In A and B, cells were given 1 mg/ml of G418 on day 1 after transduction. Cells were seeded in 24-well plates at a density of 0.5×10^6 (A, B) or 1×10^6 (C) cells per well on day 1 (A) or 2 (B and C) after transduction. A representative experiment of two individual experiments is shown in each panel.

vector paired with either pCPP (a control vector) or pCSPP (an StpC-encoding vector) shortly after transduction. Similarly, PBMCs were transduced to express Tip at a high level using pCECT, a Tip-encoding vector with EGFP, as a selection marker (data not shown).

Although transduction of PBMC using Tip-encoding vectors was very efficient (Fig. 1B) and the level of Tip expression in the transduced cells was high (Fig. 1E), we could not obtain T cells expressing Tip in a stable fashion in spite of multiple attempts. These attempts yielded numerous T-cell lines and clones expressing StpC or selection markers, but not Tip (data not shown). The conclusion from these observations was that the effect of Tip on T cells in our system could only be assessed in short-term experiments. In particular, these observations indicated that we would be unable to evaluate the ability of Tip to transform human T cells to continuous growth in culture in the absence of antigenic or mitogenic stimulation in a fashion similar to that of *H. saimiri*-induced transformation. However, because we obtained stable StpC-expressing lines that could be propagated using periodic restimulation with PHA and irradiated feeder cells, we analyzed the transformation potential of StpC in these cells. The results of a 3-month-long experiment indicated that StpC-positive T cells did not grow under conditions suitable for growth of *H. saimiri*-transformed T cells, that is, in the presence of IL-2, but in the absence of restimulations with PHA and feeders (data not shown). This result indicated that expression of StpC alone was not sufficient for immortalizing human T cells.

Tip induces apoptosis in T cells

The consistent failure to obtain stable Tip-positive T-cell lines argued that Tip might affect T-cell viability. To further analyze this phenomenon, we directly examined the effect of Tip expression as a result of lentiviral gene transfer on proliferation of anti-CD3-stimulated and control IgG-treated human T lymphocytes. This experiment showed that expression of Tip completely abrogated the low-intensity proliferation of control T lymphocytes and led to a dramatic reduction in cell numbers for anti-CD3-stimulated T lymphocytes after a short initial spike, which was still lower than the corresponding increase for T lymphocytes expressing neo alone (Fig. 2A). Thus, expression of Tip causes the eventual death of stimulated peripheral blood T lymphocytes.

The effect of Tip expression on Jurkat cells was even more profound than that on normal T lymphocytes (Fig. 2B). Furthermore, the effect of Tip on Jurkat cells was independent of external stimulation, which correlated with the continuous

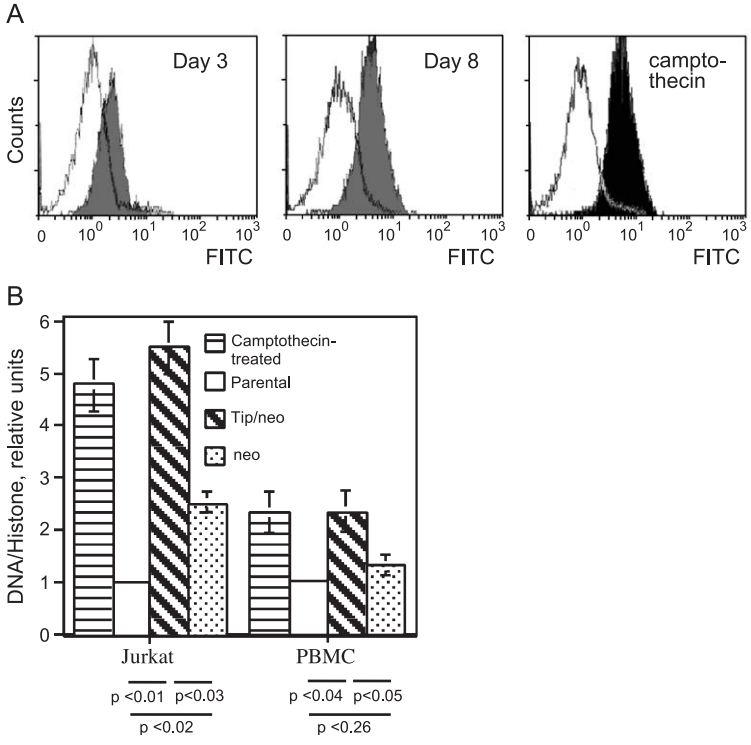

Fig. 3. Tip-induced apoptosis of T cells. (A) The TUNEL assay of Jurkat cells transduced with pCTPN to express Tip and neo (gray) or with pCPN to express neo alone (white) was performed on days 3 and 8 post-transduction. Jurkat cells treated with 5 μM camptothecin for 1 day (black) were used as a positive control. A representative of two individual experiments is shown. (B) Jurkat cells and PBMC were transduced as described in A and cultured in the presence of 1 mg/ml G418 beginning on day 1 post-transduction. Their cytoplasmic lysates were obtained 5 days after transduction and analyzed using DNA–histone ELISA. PBMCs were stimulated with surface-immobilized OKT3 on day 1 after transduction. Cytoplasmic lysates of Jurkat cells treated with 5 μM camptothecin were used as a positive control. The cytoplasmic DNA–histone level in parental cells is assigned a value of 1.00. The *P* values for the pairwise comparison of various conditions are shown at the bottom of B. A representative of three individual experiments is shown.

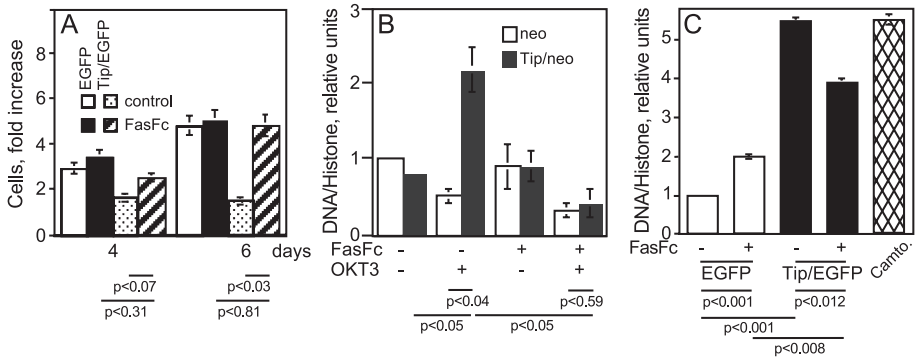

Fig. 4. Fas dependence of Tip-induced T-cell apoptosis. (A) Jurkat cells transduced with pCECT or pCE were cultured in the presence of 5 μg/ml FasFc fusion protein or an irrelevant control IgG for the time indicated, and the viable cells were counted using trypan blue exclusion. (B) PBMCs transduced with pCTPN or pCPN were activated with OKT3 or an isotype control IgG 1 day after transduction and cultured for 3 days in the presence of 1 mg/ml G418 and either 5 μg/ml FasFc fusion protein or control IgG. Cytoplasmic lysates were obtained 3 days after activation, and the amount of cytoplasmic DNA–histone complexes was determined using ELISA. (C) Jurkat cells were transduced and treated as indicated in A. Cytoplasmic lysates were obtained 3 days after transduction, and the amount of cytoplasmic DNA–histone complexes was determined using ELISA. The total number of individual experiments was three in A and two in B and C. A representative experiment is shown in each panel.

stimulation-independent growth of these cells. To exclude non-transduced, neo-negative cells from the comparison between cells expressing both Tip and neo and those expressing neo alone, we prevented growth of non-transduced cells, which could presumably be present at a low frequency, by adding G418 to the cell cultures shown in Figs. 2A and B. To confirm that the observed negative effect of Tip on cell viability was not due to the presence of G418, we carried out a similar experiment using Tip-encoding and control vectors carrying EGFP, a drug-independent selection marker, in the absence of G418. Using flow cytometry, the percentage of transduced Jurkat cells was found to be close to 100% on days 2 and 9 after transduction (data not shown). The result of this experiment was very similar to those obtained using neo-based vectors (Fig. 2C), indicating that the observed negative effect on T-cell viability was caused by Tip and not by the selection markers or the presence of G418.

To determine the mechanism of cell death induced by Tip, we examined Tip/neo- and neo-only-transduced Jurkat cells using a flow cytometry-based TUNEL assay. This experi-

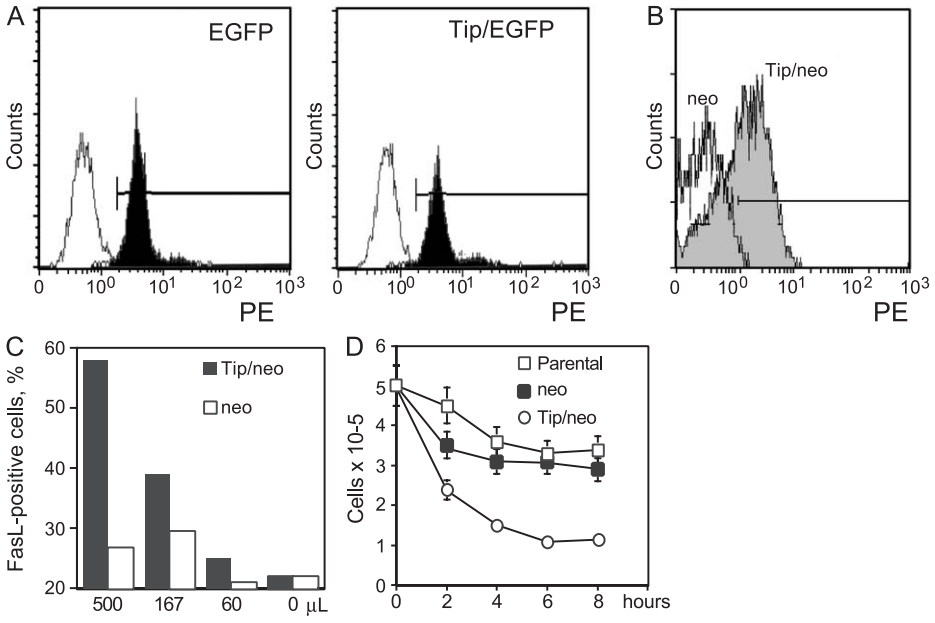

Fig. 5. Tip-induced augmentation of FasL expression and Fas-induced apoptosis. Fas (A) and FasL (B and C) expression was determined by flow cytometry on Jurkat (A and C) and PBMC (B) transduced to express Tip and selection markers or selection markers only, as indicated. TUNEL-stained (filled profiles) and untreated cells (open profiles) are shown in A. TUNEL-stained Tip/neo- and neo alone-transduced cells are shown in B as filled and open profiles, respectively. Transduction was carried out with a single dose of virus in A and B or using various doses of viral supernatants as indicated (C). (D) Jurkat cells were left intact (parental cells) or transduced with pCTPN or pCPN. Cells were seeded in an anti-Fas-coated plate to crosslink cell-surface Fas, and the numbers of viable cells were determined at various time points. Results shown in each panel are representative of three individual experiments.

ment showed that a significant fraction of Jurkat cells expressing Tip was TUNEL-positive 3 days after transduction, and that this fraction continued to grow (Fig. 3A). The level of TUNEL staining of Jurkat cells transduced to express Tip reached in these experiments that of Jurkat cells treated with camptothecin, a well-documented pharmacological inducer of apoptosis, whereas neo-transduced Jurkat cells were TUNEL-negative, demonstrating the lack of staining characteristic for untreated parental Jurkat cells (Fig. 3A). The same experiment with PBMC yielded similar results (data not shown).

These findings indicated that Tip-induced T-cell death occurred through apoptosis. To verify this conclusion, we analyzed the level of DNA–histone complexes in the cytoplasm of peripheral blood T lymphocytes and Jurkat T cells transduced with Tip/neo- or neo-alone vector. The presence of these complexes in the cytoplasm is caused by active DNA fragmentation and is indicative, like TUNEL staining, of apoptosis. These experiments demonstrated that the amount of cytoplasmic DNA–histone complex was significantly increased by Tip expression in Jurkat cells and, albeit less dramatically, in PBMC, whereas the effect of neo alone was minor (Fig. 3B).

Tip-induced T-cell apoptosis is Fas-mediated

Because the results of our experiments indicated that Tip-induced death of T cells was mediated by apoptosis, we decided to analyze the mechanism of this apoptotic death in more detail. The first issue we addressed was the role of the Fas/FasL system in Tip-induced T-cell apoptosis. The comparison of growth curves of Jurkat cells transduced with a Tip/EGFP- or EGFP-only-encoding vector in the presence of control IgG or FasFc fusion protein demonstrated that a negative effect of Tip on cell growth was abrogated by FasFc (Fig. 4A), which has been shown previously to be able to disrupt Fas/FasL interactions, thus preventing Fas-mediated cell death (Brunner et al., 1995; Ju et al., 1995). Similarly, the negative effect of Tip on CD3-induced proliferation of peripheral blood T cells (see Fig. 2A) was significantly, albeit incompletely, diminished by FasFc (data not shown).

To further evaluate the contribution of Fas/FasL interactions to Tip-induced T-cell death, we next determined the effect of FasFc fusion protein on the induction of cytosolic DNA–histone complexes by Tip. Consistent with its effect on the CD3-induced growth of peripheral blood T lymphocytes, Tip caused a dramatic increase in the level of cytosolic DNA–histone complexes in these cells, which was highly sensitive to the presence of FasFc (Fig. 4B). Likewise, FasFc significantly inhibited Tip-induced DNA fragmentation in Jurkat cells (Fig. 4C). The effect of FasFc in this case was only partial due either to the higher sensitivity of Jurkat cells to Tip-induced apoptosis or to the contribution of Fas-independent mechanisms.

Considering that the observed effects of Tip were mediated by Fas/FasL interactions, we decided to determine whether an increase in Fas or FasL expression or an increase in the sensitivity of cells to Fas-mediated signaling was the reason for the facilitation of Fas-dependent apoptosis by Tip. To evaluate the effect of Tip on the expression of Fas and FasL, we analyzed the levels of these proteins using

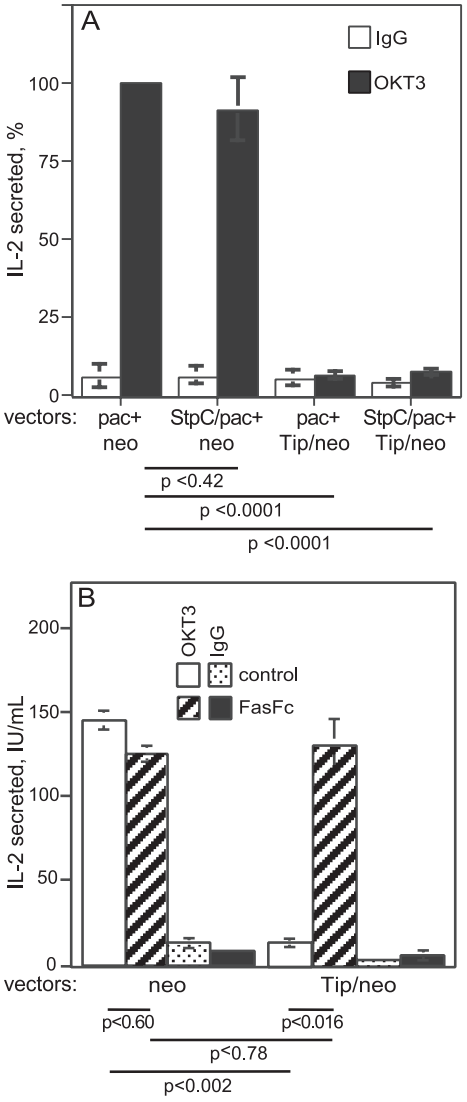

Fig. 6. Tip-induced suppression of IL-2 production by T lymphocytes. PBMCs were transduced to express Tip, StpC, or selection markers (A) or Tip or neo (B) as indicated. In A and B, cells were stimulated with anti-CD3 (OKT3) or an isotype control IgG on day 1 and cultured in the presence of 1 mg/ml G418. FasFc was added at a concentration of 5 μg/ml, where indicated (B). Supernatants were harvested 48 h after stimulation, and IL-2 was measured using sandwich ELISA. The results shown are the means and the standard errors of four independent individual experiments with different donors (A) or representative of three individual experiments (B). In A, the amount of IL-2 corresponding to 100% ranged from 60 to 170 IU/ml in individual experiments due to donor-to-donor variability. Measurements were performed in duplicates in each experiment. The *P* values for the pairwise comparison of various conditions are shown at the bottom of each panel.

Table 1
Expression of surface markers on transduced PBMC[a]

Transduction with	Surface markers[b]			
	CD3	CD4	CD8	IgG
None	79.2 (39.0)	45.4 (8.5)	20.6 (29.8)	0.8 (1.1)
neo + pac	79.8 (32.6)	47.4 (6.8)	20.4 (22.8)	1.2 (1.2)
Tip/neo + pac	81.1 (38.0)	46.8 (7.4)	20.0 (24.8)	1.1 (1.3)
StpC/pac + neo	80.0 (30.2)	47.1 (8.9)	21.1 (29.1)	1.4 (1.2)
Tip/neo + StpC/pac	81.1 (39.1)	46.3 (7.1)	20.4 (21.9)	2.1 (1.3)

[a] Cells were analyzed for surface receptor expression 48 h after transduction.

[b] Percentage of positive cells and mean fluorescent intensity of positive cells (in parentheses) are shown.

flow cytometry. These experiments indicated that Tip had no effect on Fas expression in Jurkat (Fig. 5A) or peripheral blood T cells (data not shown) while inducing an increase in the surface level of FasL on these cells (Figs. 5B and C). Detailed analysis using Jurkat cells, which were transduced at varying concentrations of the recombinant lentiviruses to cover a wide area of possible MOI values, demonstrated that an increase in the surface expression of FasL induced by Tip expression was dose-dependent, yet observed at all doses of the virus (Fig. 5C).

To evaluate the apoptotic response of T cells to Fas-mediated signaling, we crosslinked Fas on the surface of Jurkat cells using the apoptosis-inducing DX2 anti-Fas mAb immobilized on the surface of a cell culture plate. This experiment showed that Tip-expressing Jurkat cells were significantly more sensitive to Fas ligation than were parental Jurkat cells or those expressing neo alone; at 6 h after crosslinking, the number of viable cells had decreased almost 5-fold for Tip/neo-transduced cells, but only 1.5-fold for control cells (Fig. 5D).

Tip-induced apoptosis suppresses IL-2 production by T cells

Because a relationship exists between T-cell apoptosis and the ability of T cells to produce IL-2, we next analyzed the effect of Tip or StpC expression on the production of IL-2 by peripheral blood T lymphocytes. It has been shown previously that IL-2 production is increased in human T lymphocytes as a result of their *H. saimiri*-induced transformation (Biesinger et al., 1992; Broker et al., 1993; De Carli et al., 1993; Mittrucker et al., 1992, 1993; Weber et al., 1993). In contrast, our experiments demonstrated that Tip substantially

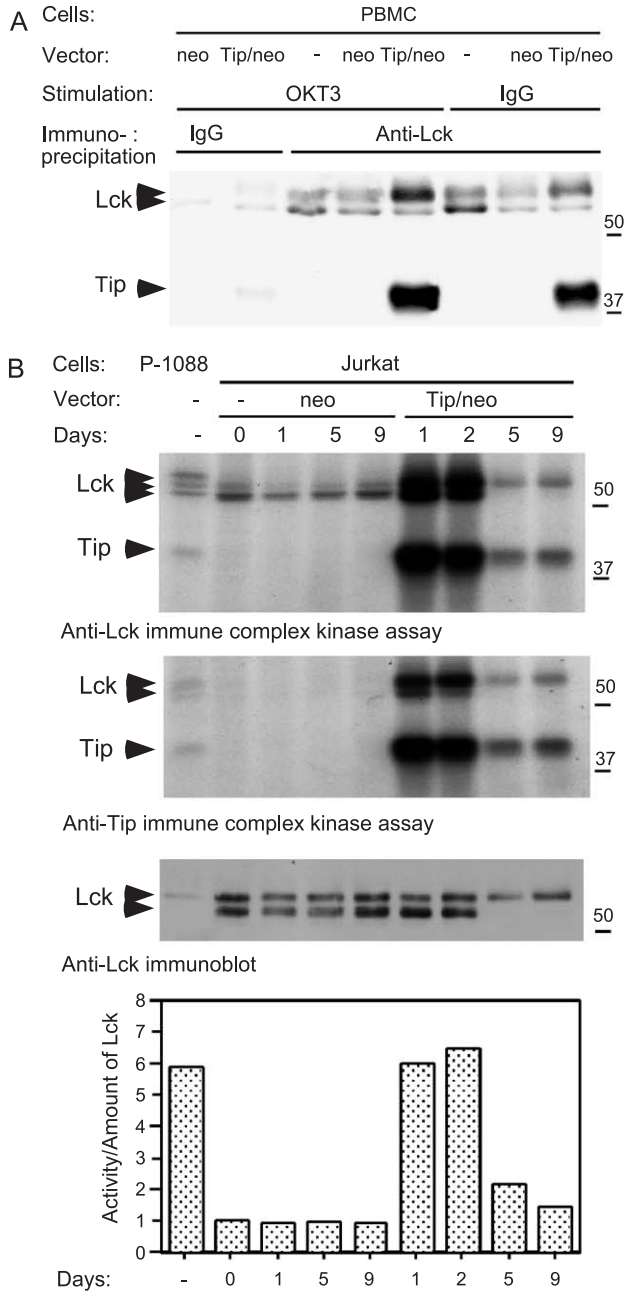

Fig. 7. Upregulation of Lck activity in Tip-expressing T cells. (A) PBMCs transduced with pCTPN or control cells (intact or transduced with pCPN) were activated with the anti-CD3 mAb OKT3 or an isotype-matching control IgG on day 1 post-transduction and lysed on day 2. Anti-Lck immune complex kinase assay was performed to determine the activity of Lck using 0.8 mg of total protein per sample. Immunoprecipitation with isotype-matching irrelevant IgG was used as a specificity control. (B) Jurkat cells transduced with pCTPN or pCPN vectors were harvested at the varying time after transduction as indicated at the top of the panel. The initial number of neo- and Tip/neo-transduced cells was adjusted to obtain equal numbers of viable cells at the time of harvesting. Anti-Tip and anti-Lck immune complex kinase assays and anti-Lck immunoblots were performed in the obtained samples using 1 mg or 50 μg of total protein per sample, respectively. P-1088 cells were used as a positive control for Tip expression. Changes in the specific activity of Lck are shown in the bottom panel of B. The specific activity of Lck is defined as its autophosphorylation intensity (determined using anti-Lck immune complex kinase assays) divided by the amount of Lck (determined using anti-Lck immunoblotting). The specific activity of Lck in parental cells is assigned the value of 1.0. The band intensities were quantified using NIH Image 1.62 software. Cells were cultured in the presence of 1 mg/ml G418 from day 1 after transduction. A representative experiment of two independent experiments is shown in A and B. The positions of Lck and Tip are indicated by arrowheads, and the positions of protein markers and their molecular masses in kDa are indicated at the right of each panel.

reduced IL-2 production by human T lymphocytes in response to CD3-mediated stimulation (Fig. 6A).

Considering that IL-2 deprivation can induce apoptosis of T cells, we examined whether the negative effect of Tip on IL-2 production causes the observed Tip-induced T-cell apoptosis by supplementing lentivirally transduced PBMC cultures with exogenous IL-2. No Tip-positive T lymphocytes were rescued from these IL-2-supplemented cultures (data not shown), arguing that T-cell apoptosis in our system was not caused by the lack of IL-2 production. This notion was supported by the findings that Jurkat cells, which lack sensitivity to IL-2, also underwent Tip-induced apoptosis in our experiments (Figs. 2–4).

We next examined the possibility that the observed decrease in IL-2 production was a consequence of the apoptotic effect of Tip. To address this issue, we treated Tip/neo- and neo-only-transduced PBMC with FasFc, as we did in the previous experiments. The results shown in Fig. 6B demonstrated that FasFc completely abolished the negative effect of Tip on IL-2 production, thus indicating that this effect, as well as those on cell viability and DNA fragmentation, was mediated by Fas-dependent apoptosis. The observed effect of Tip on IL-2 production was specific, because production of neither IL-3 nor IFN-γ was affected by Tip expression in these experiments (data not shown). These findings argued that the inhibitory effect of Tip on IL-2 production was not caused simply by a decrease in cell numbers. This conclusion is also supported by the fact that the effect of Tip on IL-2 production is observed before the onset of Tip-induced cell death (compare results shown in Figs. 2 and 6).

Finally, considering that Tip expression caused down-regulation of T-cell surface receptors in some experimental systems (Park et al., 2002), we were compelled to examine this possibility as an explanation for the observed effect of Tip on IL-2 production. Using flow cytometry, we showed that transduction of peripheral T cells with Tip or StpC did not affect expression of TCR/CD3 or its co-receptors, CD4 and CD8, in our experiments (Table 1).

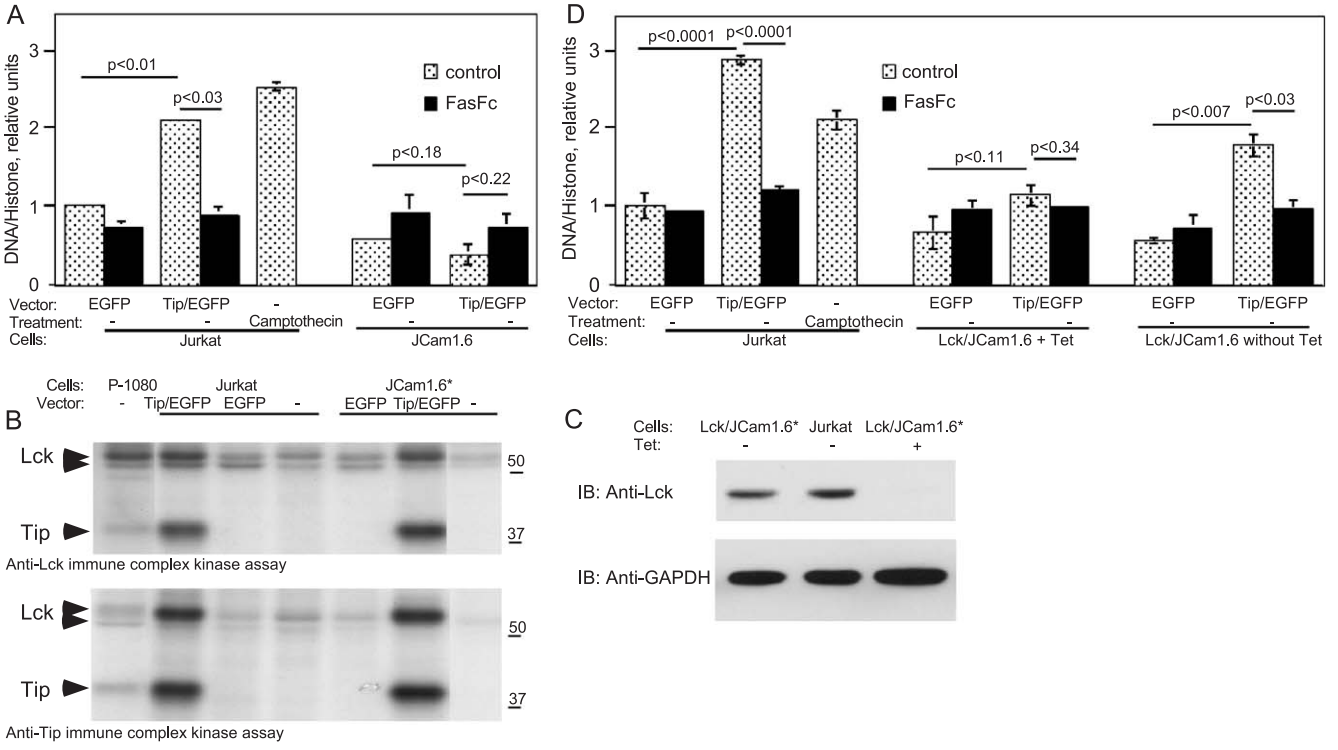

Fig. 8. Lck is essential for Tip-induced apoptosis in Jurkat cells. (A) Jurkat and J.CaM1.6 cells were left intact or transduced to express Tip or EGFP using pCECT and pCE. Five days after transduction, DNA–histone complexes were quantified in their cytoplasmic lysates. Jurkat cells treated with 5 μM camptothecin were used as a positive control. A representative experiment of three individual experiments is shown. Mean values and standard errors of duplicate measurements are shown. The amount of DNA–histone complexes in EGFP-only-transduced Jurkat cells is assigned a value of 1.00. The *P* values for the pairwise comparison of various conditions are shown inside the panel. (B) Expression of Tip was determined using anti-Tip and anti-Lck immune complex kinase assays. Lysates of parental Jurkat cells were added to the lysates of J.CaM1.6 cells as a source of Lck before immunoprecipitation. P-1080 cells were used as a positive control for Tip expression. Each lysate aliquot used as a sample or a source of Lck contained 500 μg of total protein. The positions of Lck and Tip are indicated by arrowheads, and the positions of protein markers and their molecular masses in kDa are indicated at the right of each panel. The expression results represent the experiment shown in A. (C) Expression levels of Lck were analyzed in Jurkat and Lck/J.CaM1.6 cells using immunoblotting of whole cell lysates (25 μg of total protein per lane). Tetracycline was added to Lck/J.CaM1.6 cells to repress production of Lck, where indicated. GAPDH was used as a loading control. (D) Jurkat and Lck/J.CaM1.6 cells were analyzed as described in A. Tetracycline was added to Lck/J.CaM1.6 cells to repress production of Lck, where indicated.

Tip-induced T-cell apoptosis is Lck-dependent

Physical and functional interactions between Tip and the protein tyrosine kinase Lck are well documented (see Introduction). Therefore, we were prompted to assess the effect of Tip on Lck in our experimental system and to examine the contribution of Tip/Lck interactions to the observed effects of Tip on T-cell responses. Although it has been shown previously that Tip activates Lck in multiple experimental systems (Hartley et al., 1999, 2000; Kjellen et al., 2002; Lund et al., 1997a, 1999; Wiese et al., 1996), downregulation of Lck has also been reported (Guo et al., 1997; Jung et al., 1995b). The latter could provide an explanation for the observed negative effect of Tip on IL-2 production. However, the effect of Tip on the activity of Lck in primary human T lymphocytes was found to be positive (compare Lck autophosphorylation in immune complex kinase assays shown in the lanes corresponding to Tip/neo-transduced cells to those corresponding to parental cells and cell expressing neo alone in Fig. 7A).

Because biochemical analysis of the effect of Tip on Lck required many T cells, we analyzed this effect further by transducing Jurkat T-lymphoblastoid cells to express Tip and measuring the enzymatic activity of Lck in these cells using immune complex kinase assays at various time points after transduction. These experiments indicated that the specific activity of Lck in Jurkat cells transduced to express Tip increased dramatically shortly after transduction, but rapidly declined afterwards (Fig. 7B).

Interestingly, Lck in both normal human T lymphocytes and Jurkat cells was present in at least two forms, p56 and p60 (Figs. 7A and B). The shift of the apparent molecular mass of Lck from 56 to 60 kilodaltons (kDa) has been shown to occur in activated T cells, most likely due to Erk-mediated serine phosphorylation of the unique N-terminal domain of Lck (Veillette et al., 1988; Watts et al., 1993; Winkler et al., 1993). The profound shift to the 60-kDa form was observed in both cell types examined following expression of Tip (Figs. 7A and B). This shift indicates the activated status of Tip-expressing cells and is consistent with a similar multiband pattern of Lck found in *H. saimiri*-transformed T cells (Fig. 7B).

We next evaluated the role of Lck in the apoptotic effects of Tip observed in our experimental system. To do so, we first compared the effect of Tip on DNA fragmentation in Jurkat cells, which are Lck-positive, and their J.CaM1.6 mutant, which lacks Lck (Straus and Weiss, 1992). Consistent with data shown in Fig. 4, transduction of Jurkat cells with a Tip/EGFP-encoding vector increased DNA fragmentation to a substantially higher extent than their transduction with an EGFP-only-encoding vector. Furthermore, this increase in DNA fragmentation was dependent on Fas/FasL interaction (Fig. 8A). In contrast, J.CaM1.6 cells transduced to express Tip did not show an increase in DNA fragmentation as compared to J.CaM1.6 cells transduced with a control EGFP vector (Fig. 8A). Nor did FasFc suppress Tip-induced DNA fragmentation in J.CaM1.6 cells (Fig. 8A). Differential expression of Tip could be ruled out as a possible explanation of the absence of Tip-induced apoptosis in J.CaM1.6 cells because both Jurkat and J.CaM1.6 cells expressed Tip in our experimental system (Fig. 8B). The observed differential sensitivity of Jurkat and its Lck-negative mutant J.CaM1.6 to Tip-induced apoptosis argues that the effect of Tip on Jurkat is Lck-dependent.

To confirm the role of Lck in inducing the apoptotic effects in Jurkat cells, we carried out similar experiments using Lck-reconstituted J.CaM1.6 cells (Lck/J.CaM1.6), which express Lck constitutively, but fail to express it in the presence of tetracycline (Fig. 8C). These studies demonstrated that expression of Tip caused by lentiviral transduction induces apoptosis in Lck/J.CaM1.6 cells expressing Lck, while not inducing it in Lck/J.CaM1.6 cells under conditions repressing Lck expression (Fig. 8D). These experiments also demonstrated that Tip-induced apoptosis in Lck/J.CaM1.6 cells expressing Lck is suppressed by FasFc in the same fashion as seen in Jurkat cells (Fig. 8D). Thus, the results obtained using Lck-reconstituted

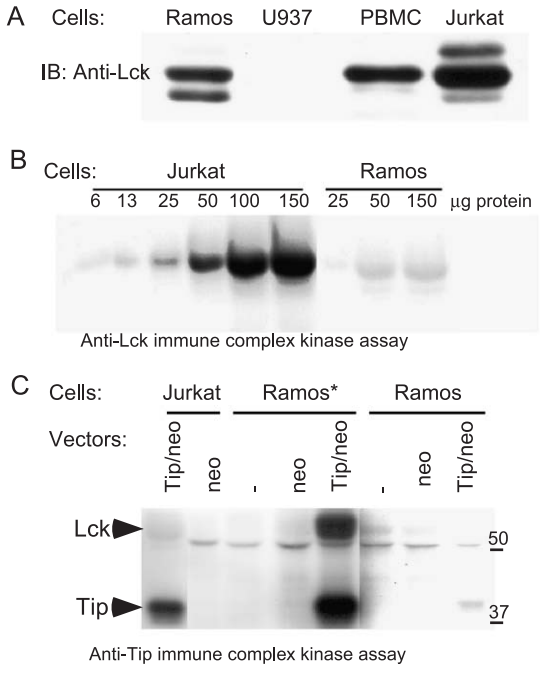

Fig. 9. The lack of Tip-mediated apoptosis in cells lacking Lck activity. (A) Expression of Lck in the B-cell line Ramos, monocytic cell line U937, T-cell line Jurkat, and freshly isolated PBMC was determined using immunoblotting of cell lysates (50 µg of total protein per lane). (B) Activity of Lck in Jurkat and Ramos cells was measured using anti-Lck immune complex kinase assay with the indicated amount of total protein. (C) Expression of Tip was determined in Jurkat and Ramos cells using anti-Tip immune complex kinase assay. Lysates of parental Jurkat cells (650 µg of total protein) were added to the lysates of Ramos cells (650 µg of total protein) as a source of Lck before immunoprecipitation. Representative experiments of at least two individual experiments are shown in each panel.

J.CaM1.6 cells indicate that the lack of Tip-induced apoptosis in J.CaM1.6 cells is not due to the deficiency of apoptotic pathways in these cells, but is caused by the lack of Lck in these cells.

To further examine the role of Lck, which is preferentially expressed in T cells, in the apoptotic effects of Tip, we attempted to transduce Tip using pCTPN and pCPN vectors into hematopoietic cells lacking Lck, such as monocytes and B cells. Using immunoblotting, we confirmed that U937, a monocytic cell line used in these experiments, was Lck-negative (Fig. 9A). In contrast, Ramos, a B-cell line, turned out to be expressing Lck, which is probably related to the transformed status of these cells (Fig. 9A). However, the activity of Lck in Ramos was virtually nil as compared to its activity in Jurkat (Fig. 9B). Therefore, both non-T cell lines tested in our experiments lacked Lck activity. In both cases, selection with G418 yielded stable Tip-expressing cell lines (Fig. 9C and data not shown), which exhibited growth rates and viability indistinguishable from those of the corresponding control and parental cell lines. The results obtained using Lck-negative non-T cell lines further argued that the apoptotic effect of Tip is mediated by Lck.

Overall, our results indicating that Tip exerts an apoptotic effect on T cells in an Lck-dependent fashion were entirely consistent with the failure to obtain Tip-positive stable T-cell lines using transduction of pHR-based Tip-encoding lentiviral vectors into normal human T lymphocytes (see above). In parallel, we carried out multiple attempts to transduce Jurkat cells to express Tip or StpC or selection markers alone using the same vectors. These experiments consistently yielded stable Jurkat cell lines following their transduction with pHR-based vectors encoding StpC plus a selection marker or a selection marker alone, but rarely following their transduction with Tip-encoding pHR-based vectors. Although these results were generally in agreement with those observed using primary T lymphocytes, we occasionally succeeded in generating stable cell lines following transduction of Jurkat cells with Tip-encoding pHR-based vectors, especially when low viral doses were used. To further analyze the dependence of Tip-mediated apoptotic effects on the presence of Lck, we examined these cell lines for the expression of Tip and Lck to determine a mechanism allowing them to survive.

The analysis of Lck expression using anti-Lck immunoblotting indicated that all stable Jurkat-based cell lines obtained in our experiments demonstrated Lck levels comparable to those in parental Jurkat cells and the *H. saimiri*-transformed T-cell line P-1088 (Fig. 10A and data not shown). However, the immune complex kinase assays showed no presence of Tip in any of the stable lines generated using pHR-based vectors, although Tip was detectable in *H. saimiri*-transformed P-1088 cells and in JTip4, a cell line generated previously from Jurkat cells using an MSCV-based vector (Merlo et al., 1998) (Fig. 10B and data not shown).

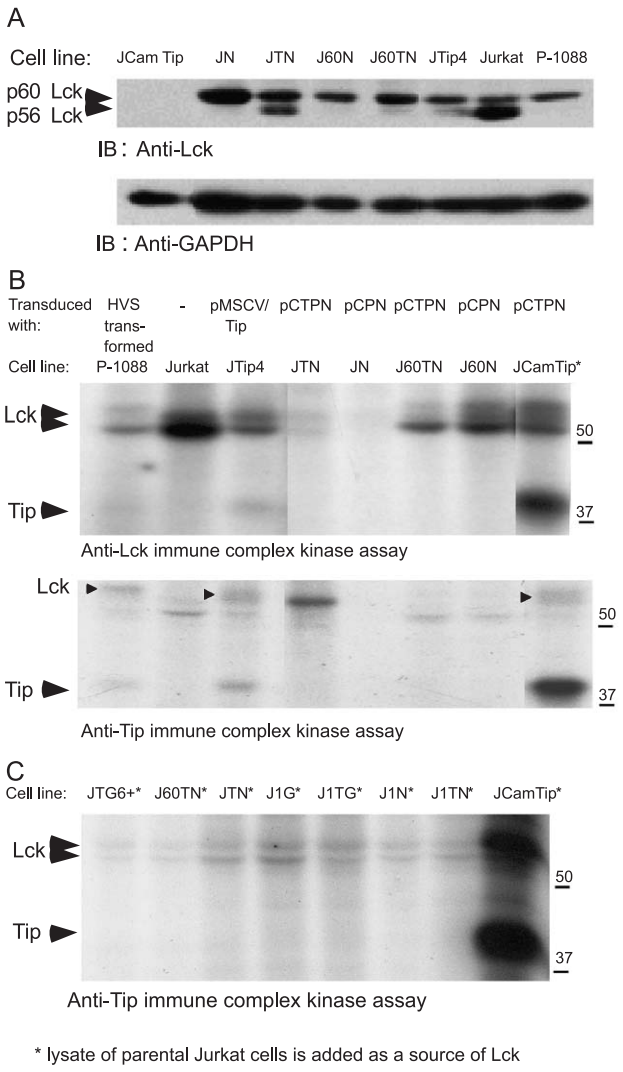

Fig. 10. Survival of Tip-negative Jurkat cells. Jurkat cells were transduced to express Tip and a selection marker or a selection marker alone as indicated at the top of each panel and sorted for EGFP expression or cultured for several weeks in the presence of 1 mg/ml G418, when cells were transduced with EGFP and neo, respectively. Stable cell lines obtained on several occasions (individual designations are indicated at the top of each panel; T, N, and G stand for Tip, neo, and EGFP, respectively) were analyzed for the expression and kinase activity of Lck using immunoblotting (A) and anti-Lck immune complex kinase assays (B), as well as for the expression of Tip using anti-Tip and anti-Lck immune complex kinase assays (B and C). Fifty, 500, and 800 μg of total protein per sample were used in A, B, and C, respectively. GAPDH was immunoblotted as loading control in A. Lysates of parental Jurkat cells (500 μg of total protein per sample) were added to the lysates of JcaM1.6-derived cells as a source of Lck before immunoprecipitation in B and to all cell lysates in C. P-1080 and P-1088 *H. saimiri*-transduced cells, as well as the Jurkat-derived JTip4 and the J.CaM1.6-derived JCamTip cell lines, were used as positive controls for Tip expression. The positions of Lck and Tip are indicated by arrowheads, and the positions of protein markers and their molecular masses in kDa are indicated at the right of the panels. The analysis of each obtained stable cell line was performed at least three times, and representative gels are shown.

To assure detection of Tip in Lck-negative cells, we employed the approach utilized to detect Tip expression in J.CaM1.6 and Ramos cells, namely, we mixed the lysates of these cell lines with the lysate of parental Jurkat cells as a source of active Lck. This experiment demonstrated the absence of Tip in all the stable Jurkat cell lines generated in our current study using pHR-based vectors (Fig. 10C). This result was clearly not due to detection failure, because Tip was easily seen in JCamTip, a Tip-positive J.CaM1.6-derived cell line, which we obtained in our current experiments (Fig. 10C). Therefore, all the examples of stable Jurkat-derived cell lines generated in this study using pCECT or pCTPP, pHR-based Tip-encoding vectors, turned out to be Tip-negative, while one of them (JTN) was found to be lacking Lck kinase activity, in addition. These results are entirely consistent with our finding that the Tip-induced Fas-mediated apoptosis of T cells is Lck-dependent.

Discussion

Previous studies have shown that Tip is capable of upregulating transcription controlled by several transcription factors (Hartley and Cooper, 2000; Hartley et al., 2000; Kjellen et al., 2002; Lund et al., 1997b, 1999; Merlo and Tsygankov, 2001) and established the essential role of Tip in T cell transformation by *H. saimiri* (Duboise et al., 1998; Medveczky et al., 1993). However, the mechanisms through which Tip participates in this transformation remain enigmatic. One of the factors restricting our understanding of the biological role of Tip is the lack of experimental systems in which the effects of Tip on normal T lymphocytes can be studied in the absence of other proteins of *H. saimiri*. To alleviate this problem, we developed a method for the efficient lentivirus-mediated introduction of Tip and StpC, another *H. saimiri* protein essential for T-cell transformation, into primary T lymphocytes using a three-vector system based on a series of HIV-based lentiviral gene transfer constructs encoding for either Tip or StpC in the presence of EGFP or a drug resistance selection marker, an HIV-based accessory construct, and a vesicular stomatitis virus glycoprotein (VSV G) pseudotyping envelope construct. Using this system, we achieved highly efficient transduction of T-lymphoblastoid cells and primary T lymphocytes in culture.

Long-term tracking of the obtained Tip- or StpC-positive primary T cells has demonstrated that expression of StpC alone cannot transform primary human T lymphocytes to antigen- or mitogen-independent continuous growth in culture. This finding is in agreement with previous reports indicating the failure of Tip-deficient *H. saimiri* to transform T cells (Duboise et al., 1998; Medveczky et al., 1993). In contrast to the definitive results demonstrating the lack of transforming effect of StpC in stable StpC-positive T-cell lines, the long-term effect of Tip was not revealed in our experiments due to the unexpected ability of Tip to induce Fas-dependent apoptosis of T cells. The apoptotic activity of Tip is evident in both T-lymphoblastoid cells and peripheral blood T lymphocytes, but in lymphoblastoid cells it appears to be constitutive, whereas in normal T lymphocytes it is increased by TCR/CD3 stimulation.

Our results indicate that Tip facilitates the surface expression of FasL (Figs. 5B and C) and the apoptotic response of T cells to Fas ligation (Fig. 5D). Both of these phenomena may be involved in Tip-induced T-cell apoptosis. It should be noted that regardless of the relative contributions of these mechanisms to the overall apoptotic effect observed in our study, Tip facilitates apoptosis not by circumventing the requirements for proper Fas/FasL interactions, but by augmenting Fas-mediated apoptotic signaling. The molecular mechanisms mediating the effects of Tip on FasL expression and the sensitivity to Fas ligation need to be elucidated further. However, it may be speculated that upregulation of FasL expression by Tip involves Tip-induced activation of NF-AT (Hartley et al., 2000; Kjellen et al., 2002; Merlo and Tsygankov, 2001), because transcription of *fasL* is known to be facilitated by NF-AT (Latinis et al., 1997). Finally, it should be noted that the pro-apoptotic effect of Tip described in this study is probably unrelated to the previously reported activation-induced Fas-independent apoptosis of *H. saimiri*-transformed T cells (Broker et al., 1997). The latter takes place in *H. saimiri*-transformed T cells and, therefore, may be a result of any combination of events that occur during cellular transformation caused by *H. saimiri*. The apoptotic effect of Tip observed in our study occurs independently of *H. saimiri* infection and does not require other *H. saimiri* proteins.

One of the most dramatic effects of Tip expression in human peripheral T lymphocytes is a decrease in IL-2 production in response to TCR/CD3-mediated stimulation, which can be prevented using soluble FasFc to block Fas/FasL interactions (Fig. 6). In spite of its relation to apoptosis, the effect of Tip on IL-2 production is not caused simply by a decrease in the number of viable cells. On the contrary, this effect of Tip is observed before the onset of Tip-induced cell death. Furthermore, this effect is specific for IL-2; at the time when a decrease in IL-2 production is clearly detected, no significant changes in either IL-3 or IFN-γ production is seen in Tip-transduced T lymphocytes (data not shown). Thus, the decrease in IL-2 production in Tip-expressing human peripheral T lymphocytes appears to be a result of the early apoptotic events caused by Tip expression.

The apoptotic effect of Tip on T cells, which we observed in our study, is clearly Lck-dependent. This conclusion is based on three complementary sets of results. First, J.CaM1.6, an Lck-negative mutant of Jurkat cells, does not show a Tip-induced increase of DNA fragmentation, while both Jurkat and Lck-reconstituted J.CaM1.6 cells demonstrate high levels of cytoplasmic DNA–histone complexes when Tip is expressed in these cells (Fig. 8). Second, cell lines expressing no active Lck can be easily transduced to express Tip in a stable fashion (Fig. 9). Third, all of the few stable Jurkat-based cell lines obtained in our experiments

following transduction with Tip-encoding lentiviral vectors turned out to be Tip-negative (Fig. 10).

The results presented in this study also allow us to clarify previously reported findings related to the functional interactions of Tip and Lck. Tip is capable of activating Lck in multiple experimental systems (Fickenscher et al., 1997; Hartley et al., 1999, 2000; Kjellen et al., 2002; Lund et al., 1997a, 1999; Wiese et al., 1996), but has also been reported to downregulate Lck in a stable Tip-positive Jurkat-based cell line (Guo et al., 1997; Jung et al., 1995b). This apparent discrepancy may be explained by the apoptotic effect of Tip/Lck interactions, which is likely to result in the selection of Tip- or Lck-deficient cells. However, the decrease in the specific activity of Lck occurring in Tip-expressing Jurkat cells between days 2 and 5 post-transduction, such as shown in Fig. 7B, is probably caused not by the gradual selection of Lck-defective cells, but by cellular mechanisms that directly regulate Lck. It is tempting to link this rapid decrease in Lck activity to the degradation of Lck induced by its targeting to lysosomes by a complex of Tip and p80 protein, which has been demonstrated recently by Park et al. (2002). However, no selective degradation of activated Lck by Tip and p80 has been shown in that study, thus making unclear the mechanism by which the specific activity of Lck can be reduced. Furthermore, the effect of Tip and p80 on Lck degradation has only been shown in Jurkat-T cells upon overexpression of both Tip and p80. Therefore, it appears more likely that the decrease in the specific activity of Lck occurring immediately after its dramatic increase caused by the expression of Tip is due to the normal cellular mechanisms downregulating activated Lck via its ubiquitin- and proteasome-dependent degradation (Rao et al., 2002). Although direct testing of this hypothesis by treatment of Tip-transduced T cells with lactacystin, a potent proteasome inhibitor, was hindered by a strong detrimental effect of lactacystin on cell viability, we demonstrated that the positive effect of Tip on Lck activity is enhanced in lactacystin-treated cells as compared to untreated cells (data not shown), thus supporting the idea that Lck activated by Tip is effectively downregulated by ubiquitin- and proteasome-dependent mechanisms.

It is important to note that any interpretation of the results presented in this report should consider the fact that the level of Tip expression in lentivirally transduced T cells was higher than that in *H. saimiri*-transformed human peripheral blood T lymphocytes. Therefore, the results of this study, while indicating that Tip has a considerable apoptotic potential, do not reflect the extent of the apoptotic effect of Tip in *H. saimiri*-transformed T cells, where Tip is expressed at a moderate level. It is likely that the peculiar mechanism of *tip* gene expression employed by *H. saimiri* (Tip is encoded by the second open reading frame of the bicistronic *stp*C/*tip* mRNA (Biesinger et al., 1990; Fickenscher et al., 1996)) evolved to reduce the cellular level of Tip to prevent excessive apoptosis. Therefore, the biological significance of the observed effect of Tip may be related to the early steps of T-cell infection by *H. saimiri*. At this stage of the viral lifecycle, Tip may be expressed at a level higher than that in *H. saimiri*-transformed cells. This notion is supported by results indicating that expression of a wide range of various *H. saimiri* genes, including the *stpC/tip* transcript, is higher in semipermissive monkey T-cell systems than in human *H. saimiri*-transformed cells, which produce no viral particles (Fickenscher et al., 1996). It is possible that the initial high-level expression of Tip causes massive death of *H. saimiri*-infected cells, hence explaining the low transformation efficiency (approximately 10^{-5}) of *H. saimiri* for human T lymphocytes (Fickenscher et al., 1997). Furthermore, the effect of Tip at early stages of viral infection can explain the presence in the genome of *H. saimiri* of two viral anti-apoptotic genes, v-FLIP (Thome et al., 1997) and a Bcl-2 homologue (Derfuss et al., 1998; Nava et al., 1997), which are not expressed in *H. saimiri*-transformed cells (Fickenscher et al., 1996; Knappe et al., 1997). The results of our study suggest that the products of these genes may be employed at the early steps of viral lifecycle to suppress the apoptotic effect of Tip, which is detrimental for cell viability and, hence, for *H. saimiri*-induced transformation. In transformed T cells, the apoptotic effect of Tip may be low due to the moderate levels of Tip expression and Lck activity in these cells and, as a result, may be effectively balanced by normal cellular anti-apoptotic mechanisms. One might also speculate that co-expression with StpC is able to diminish the apoptotic activity of Tip due to the activation of NF-κB by StpC (Lee et al., 1999; Merlo and Tsygankov, 2001). However, our results indicate that StpC exerts no protective effect on Tip-expressing T cells, either lymphoblastoid or primary (data not shown).

Finally, it should be noted that in spite of our finding that Tip demonstrated a considerable pro-apoptotic activity in T cells, it is clear that this effect is not the only one exerted by Tip on T cells because Tip is essential for *H. saimiri*-induced transformation. Instead, Tip-induced apoptosis may be considered a "side effect" of Tip on T cells, which has to be suppressed to reveal the transformation potential of Tip. The mechanisms suppressing Tip-induced apoptosis of T cells infected with *H. saimiri* remain to be elucidated further.

Materials and methods

Cells

Human Jurkat and J.CaM1.6 T-leukemic cells, Ramos B-lymphoma cells, and human renal embryocarinoma 293T were obtained from ATCC. Lck/J.CaM1.6 cells, an engineered variant of J.CaM1.6 reconstituted to express Lck in a Tet-sensitive fashion, were kindly provided by Dr. D. Straus (Virginia Commonwealth University, Richmond, VA). P-

1088 and P-1080 T-cell lines were kindly provided by Dr. B. Biesinger (Erlangen-Nurnberg University, Erlangen, Germany). Peripheral blood mononuclear cells (PBMC) were isolated from donor blood using Ficoll–Paque cushion (Amersham Pharmacia Biotech, Piscataway, NJ). Freshly isolated PBMCs contained approximately 75% T lymphocytes (defined as $CD3^+CD4^+$ plus $CD3^+CD8^+$ cells), 10% B lymphocytes (defined as $CD19^+CD22^+$ cells), and 10% monocytes/macrophages (defined as $CD14^+CD16^+$ cells). Jurkat, Lck/JCaM1.6, and J.CaM1.6 were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10 mM HEPES (Gibco/BRL, Grand Island, NY). G418 (1 mg/ml) and hygromycin B (250 μg/ml) were added to Lck/J.CaM1.6 cells. Tetracycline (1 μg/ml) was added to Lck/J.CaM1.6 cells to repress Lck expression. 293T cells were grown in DMEM (Gibco/BRL) supplemented as described above for RPMI 1640. P-1088 and P-1080 were grown in 96-well round-bottom plates in RPMI 1640 and AIM-V (Gibco/BRL) mixed at a ratio of 1:1 and containing the supplements described above, 1% human AB serum (Biocell, Carson, CA), sodium pyruvate, nonessential amino acids, and IL-2 (Eurocetus, Amsterdam, The Netherlands) at a concentration of 50 IU/ml. Long-term cultures of stable cell lines obtained from peripheral blood T-lymphocytes were propagated in 96-well plates in the same medium as for P-1080 and P-1088 lines under periodic (every 2–3 weeks) restimulation. For restimulation, cultured T cells (2×10^4 to 5×10^4 in 200 μl) were mixed with 5×10^3 irradiated human PBMC in 100 μl in the presence of PHA (Sigma; 1 μg/ml final concentration). Feeder cells were irradiated (6000 Rad) using a Cs-137 Model 30-1 γ-irradiator (J. L. Shepard and Assoc., Glendale, CA). When the ability of StpC-transduced T-cell lines to grow in the absence of periodic restimulations was studied, these cells were cultured under conditions identical to those used for P-1080 and P-1088 lines.

Plasmids

Transfer plasmids encoding for the *H. saimiri* gene of interest and capable of being packaged in retroviral particles were constructed using the pHR′-CMV-lacZ transfer vector kindly provided by Drs. I. Verma and D. Trono (Salk Institute, La Jolla, CA). The pHR′-CMV-lacZ plasmid contained the *lac*Z gene encoding for *E. coli* β-galactosidase under the control of a CMV promoter flanked with HIV LTR sequences essential for the integration of the vector into genomic DNA. The plasmid also contained a ψ-signal required for packaging and the Rev-response element facilitating transduction of quiescent cells (Naldini et al., 1996). This plasmid was used to construct pHR′-CMV-MCS, a vector containing a convenient multiple cloning site (MCS) instead of *lac*Z. To generate pHR′-CMV-MCS, we removed *lac*Z using *Bam*HI and *Xho*I and introduced an oligonucleotide containing a string of unique restriction sites (*Bam*HI, *Mlu*I, *Eco*RI, *Sal*I, *Spe*I, *Xho*I) between the sticky ends of the *Bam*HI/*Xho*I-digested pHR′-CMV-MCS. The cassette containing the Tip coding sequence and a neomycin phosphotransferase gene under the control of a murine phosphoglycerate kinase promoter (*tip*-PGK-*neo*) was excised from pMSCVneoEB/tip transfer vector using *Xho*I and *Sal*I (Fig. 1A). A similar cassette containing StpC coding sequence and the *pgk* promoter-controlled puromycin *N*-acetyl transferase gene (*stp*C-PGK-*pac*) was obtained from pMSCVpac/stpC transfer vector using *Spe*I. The MSCV-based transfect vectors used as the source of Tip and StpC coding sequences have been described previously (Merlo et al., 1998). The obtained cassettes were inserted into pHR′CMV-MCS linearized with *Nhe*I (for StpC) or *Xho*I (for Tip) to construct pHR-based plasmids containing a CMV promoter-controlled StpC or Tip coding sequence together with a PGK promoter-controlled *neo* or *pac*. These plasmids were designated CMV-Tip-PGK-Neo (pCTPN) and CMV-StpC-PGK-Pac (pCSPP). A similar strategy was used to construct control transfer plasmids without *tip* or *stpC* (pCPN and pCPP).

Enhanced green fluorescent protein (EGFP) cDNA was excised from pLEIN (Clonetech, Palo Alto, CA) using *Sal*I and *Spe*I, and inserted into pHR′CMV-MCS linearized using the same enzymes. The resulting plasmid was designated pCE. To generate a Tip expression plasmid using pCE backbone, a CMV promoter together with a Tip coding sequence was excised from the pCEP4/tip vector using *Xho*I and *Sal*I and then inserted into *Xho*I linearized pCE to yield pHR-CMV-*egfp*-CMV-*tip* (pCECT). To generate pCEP4/tip, the BglII-excised fragment of pMSCVneoEB/tip containing a Tip coding sequence was inserted into the pCEP4 vector (Stratagene, Carlsbad, CA) linearized with *Bam*HI. Similarly, a CMV-controlled StpC coding sequence was excised from pCSPP using *Cla*I and *Xho*I, and inserted into pCE linearized with *Xho*I to construct pHR-CMV-*egfp*-CMV-*stpC* (pCECS).

For the construction of pseudotyped lentiviral vectors, a transfer plasmid was used along with the packaging plasmid pCMVΔ8.5 and the envelope plasmid pMD. The packaging plasmid carries HIV *gag-pol* and *rev* and is controlled by a CMV promoter. The envelope plasmid carries the gene encoding for the vesicular stomatitis virus glycoprotein (VSV G) under the control of CMV. Both accessory plasmids have been kindly provided by Drs. I. Verma and D. Trono (Salk Institute) and are described in detail in Naldini et al. (1996).

Preparation of recombinant retrovirus and transduction

The appropriate transfer plasmid together with the packaging and envelope plasmids was transfected into 293T cells using the calcium phosphate method (Promega, Madison, WI). Supernatants containing pseudotyped retroviral vector particles were harvested and filtered

through a 0.4-μm filter 60 h after transfection and were either used immediately or frozen at −80 °C for future use. For viruses carrying drug-resistance genes, viral titers were determined using transduction of 293T adhesion cultures with varying doses of a virus followed by selection with the corresponding drug and staining with crystal violet to determine the numbers of colonies. For viruses carrying EGFP, viral titers were determined using transduction of 293T and Jurkat cells with varying doses of a virus followed by flow cytometry analysis to determine the number of EGFP-positive cells. Viral supernatants contained approximately 10^7 infectious units per ml independent of the nature of the gene transfer plasmid. To transduce T cells, viral supernatant was supplemented with 5 μg/ml polybrene (Sigma, St. Louis, MO) and added to PBMC (1 ml to 2.5×10^6 cells) or to Jurkat or J.CaM1.6 cells (0.3 ml to 1×10^6 cells) in 1-ml cultures placed in a 24-well plate. The plate was centrifuged at 800–1500 × *g* for 90 min at 32 °C. After centrifugation, the cultures were incubated at 37 °C overnight. The next day, the supernatant was replaced with fresh media, and cells were used immediately unless indicated otherwise.

Cell activation

Goat anti-mouse Fc (Cappel, Durham, NC) in PBS at a concentration of 0.1 mg/ml was added to 96-well plates at 50 μl per well or to 24-well plates at 250 μl and incubated overnight at 4 °C. The anti-CD3 mouse mAb OKT3 or an isotype-matching control mouse IgG was added to the anti-Fc-coated plates at a dose of 0.2 or 1 μg per well for 96- and 24-well plates, respectively, for 2 h at 37 °C. Transduced PBMCs were plated in the wells after unbound antibodies were washed from the wells with PBS.

Immunoblotting

Immunoblotting was performed essentially as described previously (Feshchenko et al., 1998). Briefly, cells were lysed in 1% NP-40 in Tris/NaCl/EDTA buffer with NaF, aprotinin, leupeptin, and sodium vanadate (Sigma). Equal amounts of total protein as measured using Coomasie reagent (Pierce, Rockford, IL) were treated with SDS-PAGE sample buffer, separated in SDS-PAGE gels, transferred to nitrocellulose (Amersham Pharmacia Biotech), and probed with the appropriate antibodies. For Lck, 3A5 mouse mAb (Santa Cruz Biotechnology, Santa Cruz, CA) was used. Antibodies to Tip and StpC were raised in rabbits against the corresponding recombinant GST-fusion proteins (Merlo et al., 1998). To verify equal loading rabbit polyclonal anti-GAPDH (Research Diagnostic Inc., Flanders, NJ) or anti-actin (Santa Cruz Biotechnology) was used. Protein bands were then visualized by chemiluminescence using an ECL Plus kit (Amersham Pharmacia Biotech).

Immune complex kinase assay

Immune complex kinase assays have been described previously (Feshchenko et al., 1998; Merlo et al., 1998). In short, cell lysates were immunoprecipitated with rabbit polyclonal anti-Lck or anti-Tip (Merlo et al., 1998) or control pre-immune rabbit serum. The immunoprecipitates were subjected to kinase reaction in a total of 25 μl of kinase mixture containing 20 mM MOPS (pH 7.0), 5 mM $MgCl_2$, and 1 μCi/μl [γ-^{32}P]ATP (ICN, Irvine, CA). The reaction was stopped by adding SDS-PAGE sample buffer, and the kinase mixtures were separated using SDS-PAGE. The gels were dried, and the phosphoproteins bands were visualized using autoradiography.

As described in the previous paragraph, the immune complex kinase assay could be used to determine Tip expression in cells expressing active Lck, such as Jurkats or peripheral blood T lymphocytes. To use this assay for detecting Tip in cells lacking active Lck, such as Ramos or J.CaM1.6, a Lck-negative mutant of Jurkat, we modified this technique by supplementing Lck-negative lysates with the lysate of parental Jurkat cells as a source of active Lck (Merlo et al., 1998).

Flow cytometry

To analyze T cells for EGFP expression, cells were washed once in PBS and analyzed using a Coulter EPICS Flow Cytometer (Coulter, Fullerton, CA). When required, cells were sorted using a Coulter EPICS Elite sorter. To stain for cell surface markers, cells were washed twice in PBS containing 5% fetal bovine serum and then incubated with the appropriate mouse mAb for 1 h. The primary antibody was washed off twice and replaced with PE-conjugated goat anti-mouse antibody for 30 min. The secondary antibody was washed off three times, and the cells were resuspended in PBS containing 5% fetal bovine serum. TUNEL assays were performed using an APO-Direct kit (Pharmingen, San Diego, CA) following the manufacturer's recommendations. Briefly, the cells were fixed with 1% paraformaldehyde for 15 min and permeabilized with 70% ethanol for 30 min. The cells were then incubated with FITC-labeled dUTP and TdT enzyme for 1 h, and then the excess label was washed out with PBS. The labeled cells were analyzed using flow cytometry. Both cell surface staining and TUNEL assays were carried out at 4 °C.

Apoptosis induction

To measure Fas-induced apoptosis in T cells, 250 μl of 4 μg/ml mouse antihuman Fas mAb DX2 (Pharmingen) was adsorbed for 2 h on a 24-well plate pre-coated with goat anti-mouse Fc antibody (Cappel). Jurkat cells were added to the wells (1 ml at a density of $0.5–1 \times 10^6$/ml) and centrifuged immediately at 350 × *g* for 5 min and

placed in a cell culture incubator at 37 °C. Samples of cell cultures were collected at appropriate times for the assessment of viability using trypan blue staining. To obtain a positive control for apoptosis measurements, Jurkat cells at a density of 1×10^6/ml were incubated with 5 μM camptothecin (Sigma) at 37 °C overnight and washed afterwards with PBS.

Enzyme-linked immunosorbent assays (ELISA)

Sandwich ELISA was used to determine the concentration of PBMC-secreted IL-2 and the apoptotic state of PBMC, Jurkat, or J.CaM1.6 cells. For IL-2 secretion, supernatants were incubated for 2 h with an IL-2 capture antibody, 5344.111 mouse mAb (Pharmingen), and then incubated with a biotinylated detection antibody, B33-2 mouse mAb (Pharmingen), for 1 h. Streptavidin-conjugated horseradish peroxidase (Vector Laboratories, Burlingame, CA) followed by H_2O_2 and the chromogenic peroxidase substrate 2,2′-azinibis(3-ethylbenzthiazoline-sulfonic acid) (Sigma) was added to visualize the detection antibody. A similar protocol was used to measure cytoplasmic DNA–histone complexes to assess the apoptotic process in the cells. In this case, the mouse mAbs LG11-2 and biotinylated PL2-3 (kindly provided by Drs. P. Salgame and M. Monestier of Temple University, Philadelphia, PA) were used as the capture and detection antibody, respectively. This protocol has been described in detail in Salgame et al. (1997).

Statistical analysis of data

Statistical analysis of data was carried out using the unpaired two-tail *t* test with the GraphPad Prism software (version 3.0 for Macintosh; GraphPad, San Diego, CA).

Acknowledgments

The authors would like to thank Drs. B. Biesinger, J. Merlo, M. Monestier, P. Salgame, D. Straus, D. Trono, I. Verma for kindly providing cells and reagents, Dr. P. Salgame for carefully reading the manuscript, and G. Harvey for excellent editorial help. This work was supported by the ACS grant RPG-00-105-MBC to A.Y.T.

References

Biesinger, B., Trimble, J.J., Desrosiers, R.C., Fleckenstein, B., 1990. The divergence between two oncogenic herpesvirus saimiri strains in a genomic region related to the transforming phenotype. Virology 176 (2), 505–514.

Biesinger, B., Muller-Fleckenstein, I., Simmer, B., Lang, G., Wittmann, S., Platzer, E., Desrosiers, R.C., Fleckenstein, B., 1992. Stable growth transformation of human T lymphocytes by herpesvirus saimiri. Proc. Natl. Acad. Sci. U.S.A. 89 (7), 3116–3119.

Biesinger, B., Tsygankov, A.Y., Fickenscher, H., Emmrich, F., Fleckenstein, B., Bolen, J.B., Broker, B.M., 1995. The product of the herpesvirus saimiri open reading frame 1 (tip) interacts with T cell-specific kinase p56lck in transformed cells. J. Biol. Chem. 270 (9), 4729–4734.

Broker, B.M., Fickenscher, H., 1999. Herpesvirus saimiri strategies for T cell stimulation and transformation. Med. Microbiol. Immunol. 187 (3), 127–136.

Broker, B.M., Tsygankov, A.Y., Muller-Fleckenstein, I., Guse, A.H., Chitaev, N.A., Biesinger, B., Fleckenstein, B., Emmrich, F., 1993. Immortalization of human T cell clones by herpesvirus saimiri. Signal transduction analysis reveals functional CD3, CD4, and IL-2 receptors. J. Immunol. 151 (3), 1184–1192.

Broker, B.M., Kraft, M.S., Klauenberg, U., Le Deist, F., de Villartay, J.P., Fleckenstein, B., Fleischer, B., Meinl, E., 1997. Activation induces apoptosis in herpesvirus saimiri-transformed T cells independent of CD95 (Fas, APO-1). Eur. J. Immunol. 27 (11), 2774–2780.

Brunner, T., Mogil, R.J., LaFace, D., Yoo, N.J., Mahboubi, A., Echeverri, F., Martin, S.J., Force, W.R., Lynch, D.H., Ware, C.F., et al., 1995. Cell-autonomous Fas (CD95)/Fas-ligand interaction mediates activation-induced apoptosis in T-cell hybridomas. Nature 373 (6513), 441–444.

Cabanillas, J.A., Cambronero, R., Pacheco-Castro, A., Garcia-Rodirguez, M.C., Martin-Fernandez, J.M., Fontan, G., Regueiro, J.R., 2002. Characterization of herpesvirus saimiri-transformed T lymphocytes from common variable immunodeficiency patients. Clin. Exp. Immunol. 127 (2), 366–373.

Damania, B., Jung, J.U., 2001. Comparative analysis of the transforming mechanisms of Epstein–Barr virus, Kaposi's sarcoma-associated herpesvirus, and herpesvirus saimiri. Adv. Cancer Res. 80, 51–82.

De Carli, M., Berthold, S., Fickenscher, H., Fleckenstein, I.M., D'Elios, M.M., Gao, Q., Biagiotti, R., Giudizi, M.G., Kalden, J.R., Fleckenstein, B., et al., 1993. Immortalization with herpesvirus saimiri modulates the cytokine secretion profile of established Th1 and Th2 human T cell clones. J. Immunol. 151 (9), 5022–5030.

Derfuss, T., Fickenscher, H., Kraft, M.S., Henning, G., Lengenfelder, D., Fleckenstein, B., Meinl, E., 1998. Antiapoptotic activity of the herpesvirus saimiri-encoded Bcl-2 homolog: stabilization of mitochondria and inhibition of caspase-3-like activity. J. Virol. 72 (7), 5897–5904.

Duboise, S.M., Guo, J., Czajak, S., Desrosiers, R.C., Jung, J.U., 1998. STP and Tip are essential for herpesvirus saimiri oncogenicity. J. Virol. 72 (2), 1308–1313.

Feshchenko, E.A., Langdon, W.Y., Tsygankov, A.Y., 1998. Fyn, Yes, and Syk phosphorylation sites in c-Cbl map to the same tyrosine residues that become phosphorylated in activated T cells. J. Biol. Chem. 273 (14), 8323–8331.

Fickenscher, H., Fleckenstein, B., 2001. Herpesvirus saimiri. Philos. Trans. R. Soc. Lond., B Biol. Sci. 356 (1408), 545–567.

Fickenscher, H., Biesinger, B., Knappe, A., Wittmann, S., Fleckenstein, B., 1996. Regulation of the herpesvirus saimiri oncogene stpC, similar to that of T-cell activation genes, in growth-transformed human T lymphocytes. J. Virol. 70 (9), 6012–6019.

Fickenscher, H., Bokel, C., Knappe, A., Biesinger, B., Meinl, E., Fleischer, B., Fleckenstein, B., Broker, B.M., 1997. Functional phenotype of transformed human alphabeta and gammadelta T cells determined by different subgroup C strains of herpesvirus saimiri. J. Virol. 71 (3), 2252–2263.

Gallego, M.D., Santamaria, M., Pena, J., Molina, I.J., 1997. Defective actin reorganization and polymerization of Wiskott–Aldrich T cells in response to CD3-mediated stimulation. Blood 90 (8), 3089–3097.

Guo, J., Duboise, M., Lee, H., Li, M., Choi, J.K., Rosenzweig, M., Jung, J.U., 1997. Enhanced downregulation of Lck-mediated signal transduction by a Y114 mutation of herpesvirus saimiri Tip. J. Virol. 71 (9), 7092–7096.

Hartley, D.A., Cooper, G.M., 2000. Direct binding and activation of STAT transcription factors by the herpesvirus saimiri protein Tip. J. Biol. Chem. 275 (22), 16925–16932.

Hartley, D.A., Hurley, T.R., Hardwick, J.S., Lund, T.C., Medveczky, P.G., Sefton, B.M., 1999. Activation of the lck tyrosine-protein kinase by the binding of the Tip protein of herpesvirus saimiri in the absence of regulatory tyrosine phosphorylation. J. Biol. Chem. 274 (29), 20056–20059.

Hartley, D.A., Amdjadi, K., Hurley, T.R., Lund, T.C., Medveczky, P.G., Sefton, B.M., 2000. Activation of the Lck tyrosine protein kinase by the herpesvirus saimiri Tip protein involves two binding interactions. Virology 276 (2), 339–348.

Henderson, E.E., Tsygankov, A.Y., Merlo, J.J., Romano, G., Guan, M., 1999. Altered replication of human immunodeficiency virus type 1 (HIV-1) in T cell lines retrovirally transduced to express herpesvirus saimiri proteins StpC and/or Tip. Virology 264 (1), 125–133.

Isakov, N., Biesinger, B., 2000. Lck protein tyrosine kinase is a key regulator of T-cell activation and a target for signal intervention by herpesvirus saimiri and other viral gene products. Eur. J. Biochem. 267 (12), 3413–3421.

Ju, S.T., Panka, D.J., Cui, H., Ettinger, R., el-Khatib, M., Sherr, D.H., Stanger, B.Z., Marshak-Rothstein, A., 1995. Fas(CD95)/FasL interactions required for programmed cell death after T-cell activation. Nature 373 (6513), 444–448.

Jung, J.U., Desrosiers, R.C., 1995. Association of the viral oncoprotein STP-C488 with cellular Ras. Mol. Cell. Biol. 15 (12), 6506–6512.

Jung, J.U., Trimble, J.J., King, N.W., Biesinger, B., Fleckenstein, B.W., Desrosiers, R.C., 1991. Identification of transforming genes of subgroup A and C strains of herpesvirus saimiri. Proc. Natl. Acad. Sci. U.S.A. 88 (16), 7051–7055.

Jung, J.U., Lang, S.M., Friedrich, U., Jun, T., Roberts, T.M., Desrosiers, R.C., Biesinger, B., 1995a. Identification of Lck-binding elements in tip of herpesvirus saimiri. J. Biol. Chem. 270 (35), 20660–20667.

Jung, J.U., Lang, S.M., Jun, T., Roberts, T.M., Veillette, A., Desrosiers, R.C., 1995b. Downregulation of Lck-mediated signal transduction by Tip of herpesvirus saimiri. J. Virol. 69 (12), 7814–7822.

Jung, J.U., Choi, J.K., Ensser, A., Biesinger, B., 1999. Herpesvirus saimiri as a model for gammaherpesvirus oncogenesis. Semin. Cancer Biol. 9 (3), 231–239.

Kjellen, P., Amdjadi, K., Lund, T.C., Medveczky, P.G., Sefton, B.M., 2002. The herpesvirus saimiri Tip484 and Tip488 proteins both stimulate Lck tyrosine protein kinase activity in vivo and in vitro. Virology 297 (2), 281–288.

Knappe, A., Hiller, C., Thurau, M., Wittmann, S., Hofmann, H., Fleckenstein, B., Fickenscher, H., 1997. The superantigen-homologous viral immediate–early gene Ie14/Vsag in herpesvirus saimiri-transformed human T cells. J. Virol. 71 (12), 9124–9133.

Lacey, S.F., Weinhold, K.J., Chen, C.H., McDanal, C., Oei, C., Greenberg, M.L., 1998. Herpesvirus saimiri transformation of HIV type 1 suppressive CD8+ lymphocytes from an HIV type 1-infected asymptomatic individual. AIDS Res. Hum. Retroviruses 14 (6), 521–531.

Latinis, K.M., Norian, L.A., Eliason, S.L., Koretzky, G.A., 1997. Two NFAT transcription factor binding sites participate in the regulation of CD95 (Fas) ligand expression in activated human T cells. J. Biol. Chem. 272 (50), 31427–31434.

Lee, H., Choi, J.K., Li, M., Kaye, K., Kieff, E., Jung, J.U., 1999. Role of cellular tumor necrosis factor receptor-associated factors in NF-kappaB activation and lymphocyte transformation by herpesvirus saimiri STP. J. Virol. 73 (5), 3913–3919.

Longnecker, R., Miller, C.L., 1996. Regulation of Epstein–Barr virus latency by latent membrane protein 2. Trends Microbiol. 4 (1), 38–42.

Lund, T., Medveczky, M.M., Neame, P.J., Medveczky, P.G., 1996. A herpesvirus saimiri membrane protein required for interleukin-2 independence forms a stable complex with p56lck. J. Virol. 70 (1), 600–606.

Lund, T., Medveczky, M., Medveczky, P., 1997a. Herpesvirus saimiri Tip-484 membrane protein markedly increases p56lck activity in T cells. J. Virol. 71 (1), 378–382.

Lund, T.C., Garcia, R., Medveczky, M.M., Jove, R., Medveczky, P.G., 1997b. Activation of STAT transcription factors by herpesvirus saimiri Tip-484 requires p56lck. J. Virol. 71 (9), 6677–6682.

Lund, T.C., Prator, P.C., Medveczky, M.M., Medveczky, P.G., 1999. The Lck binding domain of herpesvirus saimiri Tip-484 constitutively activates Lck and STAT3 in T cells. J. Virol. 73 (2), 1689–1694.

Martin-Villa, J.M., Ferre-Lopez, S., Lopez-Suarez, J.C., Perez-Blas, M., Castellano-Tortajada, G., Sanchez-Gomez, F., Arnaiz-Villena, A., 1998. Successful in vitro immortalization of human intestinal mucosal lymphocytes with herpesvirus saimiri. Tissue Antigens 52 (5), 430–434.

Medveczky, M.M., Geck, P., Sullivan, J.L., Serbousek, D., Djeu, J.Y., Medveczky, P.G., 1993. IL-2 independent growth and cytotoxicity of herpesvirus saimiri-infected human CD8 cells and involvement of two open reading frame sequences of the virus. Virology 196 (2), 402–412.

Meinl, E., Hohlfeld, R., 2000. T cell transformation with herpesvirus saimiri: a tool for neuroimmunological research. J. Neuroimmunol. 103 (1), 1–7.

Meinl, E., Derfuss, T., Pirzer, R., Blank, N., Lengenfelder, D., Blancher, A., Le Deist, F., Fleckenstein, B., Hivroz, C., 2001. Herpesvirus saimiri replaces ZAP-70 for CD3- and CD2-mediated T cell activation. J. Biol. Chem. 276 (40), 36902–36908.

Merlo, J.J., Tsygankov, A.Y., 2001. Herpesvirus saimiri oncoproteins Tip and StpC synergistically stimulate NF-kappaB activity and interleukin-2 gene expression. Virology 279 (1), 325–338.

Merlo, J.J., Romano, G., Gordon, S.S., Feshchenko, E.A., Peng, G., Henderson, E.E., Tsygankov, A.Y., 1998. Human T cells transduced by a retroviral vector to express herpesvirus saimiri proteins TIP and STPC. Anticancer Res. 18 (4A), 2389–2396.

Mittrucker, H.W., Muller-Fleckenstein, I., Fleckenstein, B., Fleischer, B., 1992. CD2-mediated autocrine growth of Herpes virus saimiri-transformed human T lymphocytes. J. Exp. Med. 176 (3), 909–913.

Mittrucker, H.W., Muller-Fleckenstein, I., Fleckenstein, B., Fleischer, B., 1993. Herpes virus saimiri-transformed human T lymphocytes: normal functional phenotype and preserved T cell receptor signalling. Int. Immunol. 5 (8), 985–990.

Nakamura, H., Zarycki, J., Sullivan, J.L., Jung, J.U., 2001. Abnormal T cell receptor signal transduction of CD4 TH cells in X-linked lymphoproliferative syndrome. J. Immunol. 167 (5), 2657–2665.

Naldini, L., Blomer, U., Gallay, P., Ory, D., Mulligan, R., Gage, F.H., Verma, I.M., Trono, D., 1996. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science 272 (5259), 263–267.

Nava, V.E., Cheng, E.H., Veliuona, M., Zou, S., Clem, R.J., Mayer, M.L., Hardwick, J.M., 1997. Herpesvirus saimiri encodes a functional homolog of the human bcl-2 oncogene. J. Virol. 71 (5), 4118–4122.

Park, J., Lee, B.S., Choi, J.K., Means, R.E., Choe, J., Jung, J.U., 2002. Herpesviral protein targets a cellular WD repeat endosomal protein to downregulate T lymphocyte receptor expression. Immunity 17 (2), 221–233.

Pecher, G., Harnack, U., Gunther, M., Hummel, M., Fichtner, I., Schenk, J.A., 2001. Generation of an immortalized human CD4+ T cell clone inhibiting tumor growth in mice. Biochem. Biophys. Res. Commun. 283 (4), 738–742.

Rao, N., Miyake, S., Reddi, A.L., Douillard, P., Ghosh, A.K., Dodge, I.L., Zhou, P., Fernandes, N.D., Band, H., 2002. Negative regulation of Lck by Cbl ubiquitin ligase. Proc. Natl. Acad. Sci. U.S.A. 99 (6), 3794–3799.

Saadawi, A.M., L'Faqihi, F., Diab, B.Y., Sol, M.A., Enault, G., Coppin, H., Cantagrel, A., Biesinger, B., Fleckenstein, B., Thomsen, M., 1997. Dominant clones in immortalized T-cell lines from rheumatoid arthritis synovial membranes. Tissue Antigens 49 (5), 431–437.

Saha, K., Sova, P., Chao, W., Chess, L., Volsky, D.J., 1996. Generation of CD4+ and CD8+ T-cell clones from PBLs of HIV-1 infected subjects using herpesvirus saimiri. Nat. Med. 2 (11), 1272–1275.

Saha, K., Caruso, M., Volsky, D.J., 1997. Human immunodeficiency virus type 1 (HIV-1) infection of herpesvirus saimiri-immortalized human CD4-positive T lymphoblastoid cells: evidence of enhanced HIV-1 replication and cytopathic effects caused by endogenous interferon-gamma. Virology 231 (1), 1–9.

Saha, K., Bentsman, G., Chess, L., Volsky, D.J., 1998. Endogenous produc-

tion of beta-chemokines by CD4+, but not CD8+, T-cell clones correlates with the clinical state of human immunodeficiency virus type 1 (HIV-1)-infected individuals and may be responsible for blocking infection with non-syncytium-inducing HIV-1 in vitro. J. Virol. 72 (1), 876–881.

Saha, K., Volsky, D.J., Matczak, E., 1999. Resistance against syncytium-inducing human immunodeficiency virus type 1 (HIV-1) in selected CD4(+) T cells from an HIV-1-infected nonprogressor: evidence of a novel pathway of resistance mediated by a soluble factor(s) that acts after virus entry. J. Virol. 73 (9), 7891–7898.

Salgame, P., Varadhachary, A.S., Primiano, L.L., Fincke, J.E., Muller, S., Monestier, M., 1997. An ELISA for detection of apoptosis. Nucleic Acids Res. 25 (3), 680–681.

Straus, D.B., Weiss, A., 1992. Genetic evidence for the involvement of the lck tyrosine kinase in signal transduction through the T cell antigen receptor. Cell 70 (4), 585–593.

Thome, M., Schneider, P., Hofmann, K., Fickenscher, H., Meinl, E., Neipel, F., Mattmann, C., Burns, K., Bodmer, J.L., Schroter, M., Scaffidi, C., Krammer, P.H., Peter, M.E., Tschopp, J., 1997. Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors. Nature 386 (6624), 517–521.

Tsygankov, A.Y., Romano, G., 1999. Mechanisms of cell transformation by Herpesvirus saimiri. Anticancer Res. 19 (2A), 973–983.

Veillette, A., Horak, I.D., Horak, E.M., Bookman, M.A., Bolen, J.B., 1988. Alterations of the lymphocyte-specific protein tyrosine kinase (p56lck) during T-cell activation. Mol. Cell. Biol. 8 (10), 4353–4361.

Vella, C., Fickenscher, H., Atkins, C., Penny, M., Daniels, R., 1997. Herpesvirus saimiri-immortalized human T-cells support long-term, high titred replication of human immunodeficiency virus types 1 and 2. J. Gen. Virol. 78 (Pt 6), 1405–1409.

Vella, C., Zheng, N.N., Vella, G., Atkins, C., Bristow, R.G.W., Fickenscher, H., Daniels, R.S., 1999. Enhanced replication of M-tropic HIV-1 strains in Herpesvirus saimiri immortalised T-cells which express CCR5. J. Virol. Methods 79 (1), 51–63.

Vella, C., Zheng, N.N., Easterbrook, P., Daniels, R.S., 2002. Herpesvirus saimiri-immortalized human lymphocytes: novel hosts for analyzing HIV type 1 in vitro neutralization. AIDS Res. Hum. Retroviruses 18 (13), 933–946.

Watts, J.D., Sanghera, J.S., Pelech, S.L., Aebersold, R., 1993. Phosphorylation of serine-59 of p56(lck) in activated T-cells. J. Biol. Chem. 268 (31), 23275–23282.

Weber, F., Meinl, E., Drexler, K., Czlonkowska, A., Huber, S., Fickenscher, H., Muller-Fleckenstein, I., Fleckenstein, B., Wekerle, H., Hohlfeld, R., 1993. Transformation of human T-cell clones by Herpesvirus saimiri: intact antigen recognition by autonomously growing myelin basic protein-specific T cells. Proc. Natl. Acad. Sci. U.S.A. 90 (23), 11049–11053.

Wiese, N., Tsygankov, A.Y., Klauenberg, U., Bolen, J.B., Fleischer, B., Broker, B.M., 1996. Selective activation of T cell kinase p56lck by Herpesvirus saimiri protein Tip. J. Biol. Chem. 271 (2), 847–852.

Winkler, D.G., Park, I., Kim, T., Payne, N.S., Walsh, C.T., Strominger, J.L., Shin, J., 1993. Phosphorylation of Ser-42 and Ser-59 in the N-terminal region of the tyrosine kinase p56(lck). Proc. Natl. Acad. Sci. U.S.A. 90 (11), 5176–5180.

Zheng, N.N., Vella, C., Easterbrook, P.J., Daniels, R.S., 2002. Selection following isolation of human immunodeficiency virus type 1 in peripheral blood mononuclear cells and herpesvirus saimiri-transformed T cells is comparable. J. Gen. Virol. 83 (Pt 6), 1343–1352.