

# Inhibition of TCR Signaling by Herpes Simplex Virus<sup>1</sup>

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T lymphocytes are an essential component of the immune response against HSV infection. We previously reported that T cells became functionally impaired or inactivated after contacting HSV-infected fibroblasts. In our current study, we investigate the mechanisms of inactivation. We report that HSV-infected fibroblasts or HSV alone can inactivate T cells by profoundly inhibiting TCR signal transduction. Inactivation requires HSV penetration into T cells but not de novo transcription or translation. In HSV-inactivated T cells stimulated through the TCR, phosphorylation of Zap70 occurs normally. However, TCR signaling is inhibited at linker for activation of T cells (LAT) and at steps distal to LAT in the TCR signal cascade including inhibition of calcium flux and inhibition of multiple MAPK. Inactivation of T cells by HSV leads to the reduced phosphorylation of LAT at tyrosine residues critical for TCR signal propagation. Treatment of T cells with tyrosine phosphatase inhibitors attenuates inactivation by HSV, and stimulus with a mitogen that bypasses LAT phosphorylation overcomes inactivation. Our findings elucidate a potentially novel method of viral immune evasion that could be exploited to better manage HSV infection, aid in vaccine design, or allow targeted manipulation of T cell function. *The Journal of Immunology*, 2006, 176: 1825–1833.

When T lymphocytes are triggered through the TCR, they elicit powerful immune responses that include production of cytokines and destruction of cells infected with intracellular pathogens. The cytotoxic capacity of T cells is a critical host defense mechanism against most viral infections, including members of the herpesviridae family. For example, the appearance of HSV-specific T cells with cytolytic function against HSV-infected cells corresponds well with viral clearance from lesions (1, 2), and adoptive transfer studies suggest that HSV-specific T cells can protect against HSV challenge in mice (3). Despite the presence of HSV-reactive T cells, HSV causes substantial human morbidity. The seroprevalence rate of HSV-1 in the United States approaches 90% in certain populations (4), and HSV-2 is the leading cause of genital ulcers worldwide (5). One-third of those infected with HSV experience recurrent, painful mucocutaneous ulcers as a result of viral reactivation (6). Although uncommon, more serious complications of HSV infection include keratitis, the leading cause of corneal blindness in the developed world (7), fatal sporadic encephalitis (8), and disseminated multi-organ disease in immunocompromised individuals (9). In addition, a synergistic relationship exists between HSV infection and transmission of HIV (10).

Although primary infection with HSV elicits cellular and humoral responses, neither HSV-specific T cells nor neutralizing Abs are sufficient to prevent recurrence of HSV (11). This suggests that immune modulation strategies may be crucial in the life cycle of HSV. Given the role of T cells in controlling HSV infection, it is perhaps not surprising that HSV has evolved mechanisms to specifically evade and inhibit T cell function. For example, the ICP47 protein of HSV inhibits the TAP-mediated loading of peptides on MHC class I (12). This leads to down-regulation of MHC on the surface of HSV-infected cells and to decreased CTL killing in vitro (13, 14). If HSV-specific CTL do recognize HSV-infected cells, the CTL release granules that induce apoptosis in the infected target cell. To inhibit this pathway, the HSV gene products of U<sub>S</sub>3 and U<sub>S</sub>5 inhibit apoptosis induced by CTL via the granzyme B and Fas pathways (15, 16).

Although HSV-infected cells can evade T cell recognition and inhibit T cell-induced apoptosis, they are eventually recognized and removed with the help of HSV-specific T cells. To do this, T cells must ultimately be triggered through the TCR. TCR ligation initiates a signal that propagates from the plasma membrane to the T cell nucleus. A driving force in TCR signal transduction is protein phosphorylation, mediated in part by protein tyrosine kinases (17, 18). Proximal events in the TCR cascade lead to the phosphorylation and activation of Lck and Zap70. Once activated, Lck and Zap70 phosphorylate linker for activation of T cells (LAT),<sup>3</sup> an adapter molecule that anchors multiple signaling complexes (19). Tyrosine phosphorylation of LAT is required for downstream events, such as calcium mobilization and activation of MAPK in the Ras pathway (20). These events ultimately lead to release of cytotoxic granules and increased cytokine synthesis. When activated through the TCR, T cells have the ability to destroy healthy host tissue as well as infected cells. Therefore, activation of T cells through the TCR is tightly regulated. One method of TCR signal regulation occurs through the balance of phosphorylation and dephosphorylation reactions within the T cell, i.e., protein tyrosine

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<sup>3</sup> Abbreviations used in this paper: LAT, linker for activation of T cells; EB, entry blocking; MOI, multiplicity of infection; p, phosphorylated; HVEM, herpesvirus entry mediator; ActD, actinomycin D; CHX, cycloheximide; TPI, tyrosine phosphatase inhibitor.

phosphatases are thought to offset the action of protein tyrosine kinases (21).

Our laboratory has previously reported that contact between CTL and HSV-infected fibroblasts inhibited CTL cytolytic and cytokine effector functions, a process we have termed inactivation (22). In this report, we demonstrate that the inactivation of T cells by HSV requires viral penetration but not viral gene expression. Furthermore, we report that HSV inhibits TCR signaling machinery at the level of LAT, perhaps in part due to reduced tyrosine phosphorylation of LAT. The inactivation of T cells by HSV may represent a previously unrecognized mechanism of viral-induced immune regulation, whereby entry of HSV protein into T cells profoundly inhibits TCR signal transduction by altering the tyrosine phosphorylation equilibrium.

## Materials and Methods

### Cell lines and viruses

The HLA-A3-restricted CD8<sup>+</sup> CTL clone KSN, provided by E. H. Warren (Fred Hutchinson Cancer Research Center, Seattle, WA), was stimulated using a 14-day schedule as previously described (23). The Jurkat immortalized T cell clone E6-1, purchased from American Type Culture Collection (ATCC), was maintained between  $2 \times 10^5$  and  $1 \times 10^6$  cells/ml in RPMI 1640 supplemented with 4 mM HEPES, 3 mM L-glutamine, 10% FCS. Human fibroblasts, obtained from human foreskin samples, were used from passages 5 to 12 and maintained in DMEM, 10% FCS, 50 U/ml penicillin, and 50  $\mu$ g/ml streptomycin. Vero cells, used to propagate and titer HSV unless noted otherwise, were obtained from ATCC and grown in the same medium as fibroblasts. Cells were screened regularly for mycoplasma by the Biologics Production Facility at the Fred Hutchinson Cancer Research Center.

The wild-type viruses HSV-1 (strain F), HSV-2 (HG52), and the gJ deletion mutant (RAS116) were previously described (22). HSV-1 (KOS), obtained from J. A. Blaho (Mount Sinai School of Medicine, New York, NY), was originally from S. Silverstein (Columbia University, New York, NY). The HSV-1 (F) gD deletion mutant (vRR1097), originally from R. J. Roller (University of Cambridge, Cambridge, U.K.) (24), the HSV-1 (SC16) gH mutant (SCgHZ), originally from T. Minson (University of Iowa, Iowa City, IA) (25), and the HSV-1 (F) gE and gI deletion mutants (F-gE-GFP and F-gE-GFP) (26) were provided by D. C. Johnson (Oregon Health and Science University, Portland, OR). vRR1097 was grown on the gD-complemented cell line VD60 (27), and SCgHZ was grown on the gH-complemented cell line F6 (25). The HSV-1 (KOS) mutants deleted for gB (K082), originally obtained from N. A. DeLuca (University of Pittsburgh School of Medicine, Pittsburgh, PA) (28), gL (gL86) (29), and gC  $\Delta$ C2-3 (30) were provided by P. G. Spear (Northwestern University, Chicago, IL). Complemented mutant viral stocks had at least a  $10^4$ -fold differential when titered on their complementing cell lines compared with Vero cells. The HSV-1 (KOS) mutants,  $\Delta$ 7-21 (31), and D30P (32), have mutations in gD that abolish interactions with HVEM but not nectin-1. These mutants retain the ability to replicate in Vero cells.

### Abs and the HSV entry blocking (EB) peptide

The anti-CD3 mAb, OKT3 (Ortho McNeil), was used to trigger TCR signal transduction in primary CTL and Jurkat T cells. For detection of cytokine production by flow cytometry, PE-conjugated anti-IFN- $\gamma$  clone 25723.11 (BD Biosciences) was used. For detection of phosphorylation events following TCR stimulation by flow cytometry and Western immunoblotting, rabbit-polyclonal Abs against phospho-Zap70 (Tyr<sup>319</sup>)/Syk (Tyr<sup>352</sup>), phospho-LAT (Tyr<sup>191</sup>), phospho-LAT (Tyr<sup>171</sup>), phospho-MEK (Ser<sup>217/221</sup>), phospho-ERK (Thr<sup>202</sup>/Tyr<sup>204</sup>), and phospho-JNK (Thr<sup>183</sup>/Tyr<sup>185</sup>) were obtained from Cell Signaling Technology. Rabbit polyclonal Abs against phospho-LAT (Tyr<sup>132</sup>) and phospho-LAT (Tyr<sup>226</sup>) were obtained from AbCam. mAb clone 45 (BD Pharmingen) was used to detect the 42-kDa alternate transcript of total LAT. mAb clone 11B.12 (Upstate Biotechnology) was used to detect the 36- and 38-kDa transcripts of total LAT. The secondary Ab used for flow cytometry was donkey anti-rabbit FITC-conjugated (Jackson ImmunoResearch Laboratories). For immunoblotting, either goat-anti-rabbit or goat-anti-mouse IgG conjugated to HRP secondary Abs were used (Cell Signaling Technology).

The EB (for HSV EB) peptide was used to inhibit fusion of preadsorbed HSV into T cells (33). The EB peptide (RRKKAALLPAVLLALLAP) was synthesized at the Fred Hutchinson Cancer Research Center Proteomics shared facility using an automated 12-channel Rainin-PTI Symphony

instrument. The peptide was desalted (Sephadex). Peptide purity was determined by analytical HPLC, and the molecular mass was determined by electrospray ionization mass spectroscopy.

### Cytokine production

Detection of intracellular IFN- $\gamma$  production in CTL was done as previously described (22) with the following exceptions. Fibroblasts were infected overnight at a multiplicity of infection (MOI) of 10 in the presence of 50  $\mu$ M acyclovir. CTL were inactivated by coincubation with HSV-infected fibroblasts for 6 h, removed, and stimulated with immobilized anti-CD3 mAb for 6 h, the final 4 h in the presence of brefeldin A. OKT3 was immobilized on tissue culture wells at 10  $\mu$ g/ml in PBS for 16 h at 37°C.

Detection of secreted IL-2 by Jurkat T cells was as follows. Jurkat T cells were inactivated as described above for CTL. TCR stimulation was done with immobilized OKT3 at 10  $\mu$ g/ml for 24 h at 37°C without brefeldin A. Production of secreted IL-2 by Jurkat T cells was detected in triplicate by ELISA (Fred Hutchinson Cancer Research Center core facility).

### Calcium flux

Jurkat T cells were inactivated on HSV-infected fibroblasts as described above. Alternatively, T cells were treated with UV-inactivated HSV. UV treatment was performed in a Stratalinker (1 joule) on ice, and inhibition of viral gene expression was verified by titer reduction (our unpublished observations). T cells were then incubated in culture medium with 2  $\mu$ M of the calcium-binding dye indo-1 AM (Molecular Probes) for 30 min at 37°C with agitation halfway through dye loading. Cells were then washed twice, once in culture medium and once in calcium buffer (HBSS containing 1 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>, 0.5 mM Mg SO<sub>4</sub>, and 0.5% BSA) before incubating in calcium buffer for 15 min at 37°C to allow de-esterification of indo-1 AM. Samples were analyzed on a LSR1 flow cytometer (BD Biosciences) to obtain a baseline, stimulated with OKT3 at 3  $\mu$ g/ml, and immediately placed back on the cytometer. A plot of the 400–510 nm fluorescence ratio vs time (CellQuest software; BD Biosciences) was used to determine the percentage of cells that fluxed calcium.

### Transfection of Jurkat T cells with a HSV-reporter construct

To detect entry of HSV protein into T cells, we used pMLP01, a plasmid having the *Escherichia coli lacZ* gene under control of the HSV-1 ICP4 promoter and expressing  $\beta$ -galactosidase upon infection of cells with HSV (29). Transfection was done with 2.4  $\mu$ l of DMRIE-C lipid transfection reagent, 1.6  $\mu$ g of plasmid, and  $8 \times 10^5$  cells as per manufacturer's protocol (Invitrogen Life Technologies). Forty-eight hours posttransfection, cells were inactivated on HSV-infected fibroblasts in the presence of acyclovir, formalin-fixed, and stained with X-gal to visualize the HSV VP16 protein as previously described (29). Typical transfection efficiencies in Jurkat T cells using this protocol are 5% at 48 h using the pEGFP plasmid (our unpublished observations).

### Detection of phosphoproteins

CTL or Jurkat T cells were inactivated and either not stimulated or stimulated with OKT3. Cells were incubated with OKT3 at 10  $\mu$ g/ml for 15 min on ice followed by cross-linking with goat-anti-mouse Ab at 15  $\mu$ g/ml for 15 min on ice. Cells were then incubated at 37°C for 10 min for phosphorylated ERK (pERK), for 5 min for pMEK and pJNK, or for 2 min for pZap70 and pLAT. As an alternative to TCR stimulation, cells were treated with 20 ng/ml PMA plus 1  $\mu$ g/ml ionomycin (Sigma-Aldrich).

For flow cytometric analysis following TCR stimulation, cells were then fixed with 2% paraformaldehyde for 10 min at 37°C followed by drop-wise addition of ice-cold methanol to a final concentration of 90%. Cells were then incubated on ice for 30 min, washed once with buffer (PBS, 0.8% BSA), and incubated with the primary Ab at a 1/100 dilution for 30 min at 25°C. Cells were washed once and incubated with secondary Ab at a 1/200 dilution for 30 min at 25°C in the dark. Cells were washed once and analyzed on a FACSCalibur flow cytometer (BD Biosciences).

For detection of phosphoproteins by Western immunoblotting following stimulation, cells were then lysed for 30 min on ice in lysis buffer (1% Nonidet P-40, 0.25% sodium deoxycholate, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EGTA, 1 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM PMSF, 1  $\mu$ g/ml *N*-tosyl-L-phenylalanine chloromethylketone, and 1  $\mu$ g/ml *N*- $\alpha$ -p-tosyl-L-lysine chloromethylketone). Nuclear and cellular debris were removed by centrifugation, and cell lysates were boiled in denaturation buffer (55% sucrose, 20% SDS, bromophenol blue, 2-ME, 1M Tris-HCl (pH 7)) for 5 min. Lysates were separated on 4–12% SDS-PAGE gels (Invitrogen Life Technologies), transferred to nitrocellulose membranes, and blocked with PBS/5% milk. Membranes were probed with primary Abs following

the manufacturer's suggested protocol followed by detection with HRP-conjugated secondary Ab. Signals were examined with the SuperSignal chemiluminescent substrate (Pierce).

## Results

### *Jurkat T cells inactivated by HSV-infected fibroblasts have reduced cytokine synthesis and reduced calcium mobilization following TCR stimulation*

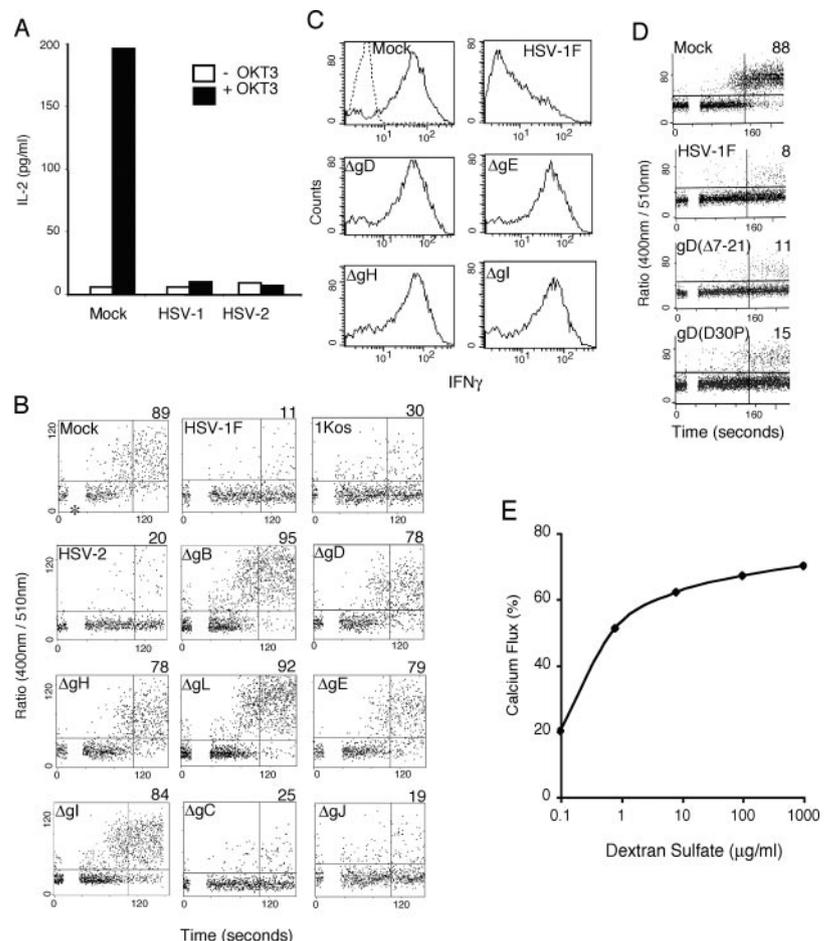
Jurkat T cells are an immortalized T cell line used to study TCR signaling events (34). Jurkat T cells were incubated on mock-infected fibroblasts or HSV-infected fibroblasts and stimulated with immobilized OKT3 mAb to induce signaling through the TCR (35). We have previously reported that primary CTL inactivated by HSV-infected fibroblasts had reduced IFN- $\gamma$  synthesis in response to TCR stimulation (22). Jurkat T cells inactivated by HSV-infected fibroblasts also had marked inhibition in cytokine synthesis (IL-2) following TCR ligation (Fig. 1A). IL-2 was analyzed instead of IFN- $\gamma$  in Jurkat T cells due to vastly superior response levels (36). We next examined whether HSV-inactivated T cells could flux calcium in response to TCR stimulation. In Jurkat T cells incubated on mock-infected fibroblasts, TCR stimulation rapidly increased intracellular calcium mobilization (Fig. 1B). However, TCR stimulation did not lead to increased calcium flux in T cells inactivated by fibroblasts infected with HSV-1 or HSV-2 (Fig. 1B). These findings demonstrate that TCR signal transduction and downstream effector function are profoundly inhibited in Jurkat T cells inactivated by HSV-infected fibroblasts.

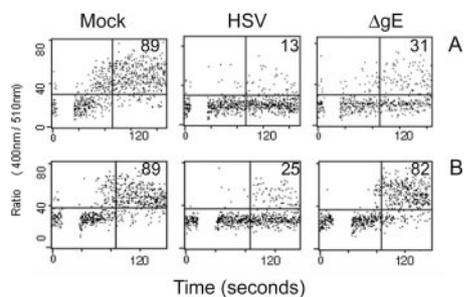
### *HSV-infected fibroblasts require viral glycoproteins involved in entry to inactivate T cells*

To assess the role of viral entry in inactivation, we examined whether fibroblasts infected with various HSV glycoprotein deletion mutants could inactivate T cells. Fusion of the viral envelope with the cellular plasma membrane requires the HSV glycoproteins gD, gB, gH, and gL (25, 27). The essential glycoprotein deletion viruses used in these experiments were complemented. A complemented virus contains the glycoprotein in its envelope and is capable of one round of infection in fibroblasts but produces progeny that lack the essential glycoprotein and, thus, cannot spread to T cells. Fibroblasts infected with wild-type HSV-1 or HSV-2 were capable of inactivation, as demonstrated by reduced calcium flux in Jurkat T cells (Fig. 1B) and by reduced cytokine (IFN- $\gamma$ ) synthesis in primary T cells (Fig. 1C). In contrast, fibroblasts infected with HSV mutants containing deletions in gB, gD, gH, or gL could not inactivate T cells (Fig. 1, B and C).

HSV mutants lacking gB, gD, gH, or gL (glycoproteins all required for viral entry) could not inactivate T cells. This indicated that HSV entry into T cells might be required for inactivation. Alternatively, the cellular receptors that bind these glycoproteins might transmit an inactivating signal to T cells. To distinguish these possibilities, we examined the role of two additional HSV glycoproteins that enhance cell-to-cell transmission of HSV, gE and gI (37, 38). As seen with the essential glycoprotein mutants, fibroblasts infected with the HSV gE or gI deletion mutants did not inactivate T cells (Fig. 1, B and C). In contrast, deletions of glycoproteins that do not play a role in HSV entry or cell-to-cell viral

**FIGURE 1.** Inactivation of T cells by HSV-infected fibroblasts requires HSV glycoproteins involved in viral entry. *A*, Jurkat T cells were incubated with fibroblasts (mock) or HSV-infected fibroblasts (HSV-1 or HSV-2), removed, and TCR stimulated with mAb against CD3 (OKT3). Secreted IL-2 was detected by ELISA. *B*, Jurkat T cells were incubated with HSV-infected fibroblasts or with fibroblasts infected with various HSV glycoprotein deletion mutants ( $\Delta$ ). Calcium flux was assessed by staining with indo-1 and analysis was done by flow cytometry. Numbers indicate the percentage of T cells that mobilized calcium in response to TCR stimulation with OKT3. OKT3 was added at the time indicated by an asterisk (\*, seen as a gap in the tracing). *C*, CTL were incubated with HSV-infected fibroblasts or with fibroblasts infected with HSV glycoprotein deletion mutants. CTL were TCR-triggered (solid lines) or untriggered (dashed line), stained for intracellular IFN- $\gamma$ , and analyzed by flow cytometry. *D*, Jurkat T cells were incubated on HSV-infected fibroblasts or incubated with the HSV gD mutants,  $\Delta 7-21$  and D30P, or (*E*) Jurkat T cells and HSV-infected fibroblasts were pretreated for 30 min with dextran sulfate before co-culturing for 4 h. Calcium flux was measured as described above. Results shown are representative of three independent trials.





**FIGURE 2.** T cells are inactivated by direct infection with HSV and  $\Delta gE$ . *A*, Jurkat T cells were infected directly with HSV at an MOI of 25 for 6 h in the presence of 50  $\mu M$  acyclovir. *B*, Alternatively, Jurkat T cells were inactivated on HSV-infected fibroblasts as described in Fig. 1*A*. The percentage of T cells able to flux calcium was assessed as described in Fig. 1. Results shown are representative of three independent trials.

spread, gC or gJ, had no effect on inactivation (Fig. 1*B*). T cell inactivation by HSV-infected fibroblasts requires HSV glycoproteins involved in envelope fusion and cell-to-cell viral transmission, while glycoproteins that are not required for viral entry are not required for inactivation. These results suggest that inactivation involves HSV entry into T cells.

#### *Inactivation requires viral adsorption but not binding to HVEM*

The role of the gD ligand in HSV fusion to the plasma membrane is mediated by binding to three classes of cell surface receptors that include the herpesvirus entry mediator (HVEM or HveA). To determine whether inactivation involved HVEM ligation, we investigated whether HSV gD mutants unable to bind HVEM,  $\Delta 7-21$  and D30P (31, 32), could inactivate T cells. We found that these mutants inactivated Jurkat T cells as well as wild-type HSV

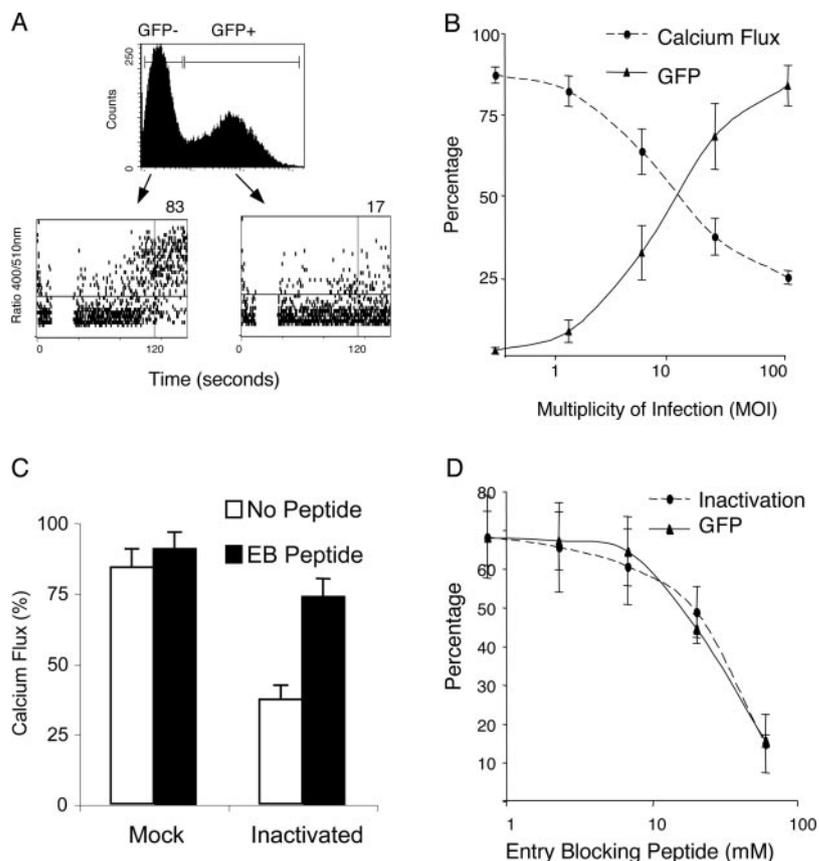
(Fig. 1*D*). These results demonstrated that ligation of HVEM on T cells is not required for inactivation.

HSV entry is initiated by an adsorption step that involves binding of the HSV glycoproteins gB and gC to heparan sulfate, a glycosaminoglycan found on many cell surface proteoglycans. Dextran sulfate, a homolog of heparan sulfate, interferes with HSV adsorption and effectively prevents penetration of HSV into cells (39). When HSV-infected fibroblasts were pretreated with dextran sulfate before incubation with T cells, dextran sulfate effectively inhibited inactivation. As levels of dextran sulfate increased, calcium flux increased in a dose-dependent manner (Fig. 1*E*). This result suggests that adsorption of HSV onto T cells is necessary for inactivation.

#### *Inactivation of T cells by direct infection with HSV does not require gE*

If viral adsorption and fusion is required for inactivation, then direct infection of T cells with HSV might also lead to inactivation. gE is involved in the spread of HSV from cell to cell, but it does not play a role in a direct viral entry (37, 38). Thus, if inactivation requires HSV entry, direct infection with HSV would likely not require gE. Indeed, direct infection with both the wild-type HSV and the gE deletion mutants ( $\Delta gE$ ) inhibited calcium flux (Fig. 2*A*). In contrast, T cells incubated with fibroblasts infected with wild-type HSV but not  $\Delta gE$  had reduced calcium flux following TCR stimulation (Fig. 2*B*). Similar results were obtained with the  $\Delta gI$  mutant (our unpublished observation). These experiments demonstrate that HSV alone can inhibit TCR signaling in T cells and that HSV glycoproteins involved in cell-to-cell spread are not required for inactivation in a direct infection model.

**FIGURE 3.** Penetration of HSV into T cells is required for inactivation. Jurkat T cells were directly infected with a HSV-1 construct that expresses GFP upon entry and viral gene expression. Cells were infected at an MOI of 25 or at the MOI indicated (*B*) for 5 h. Acyclovir was not included, as this prevented GFP expression. Cells were stained with indo-1 AM and calcium flux was determined as previously described. *A*, Events were gated as GFP negative (GFP<sup>-</sup>) or positive (GFP<sup>+</sup>) before determining the percentage of cells that fluxed calcium. Alternatively, cells were not gated before assessing calcium flux (*B-D*). HSV was preadsorbed on T cells at 4°C for 45 min before adding EB peptide at 50  $\mu M$  (*C*) or at the concentrations indicated (*D*). Cells were incubated for 45 additional min at 4°C, warmed to 37°C to allow entry, and incubated for 5 h before indo-1 AM staining. An irrelevant peptide had no effect on GFP expression or inactivation, and EB peptide was not virucidal i.e., there was no loss in plaque number when HSV was pretreated with 50  $\mu M$  EB peptide, serially diluted, and grown on Vero cells (our unpublished observations). Results represent the average of three independent trials.



### Inactivation requires HSV penetration into T cells

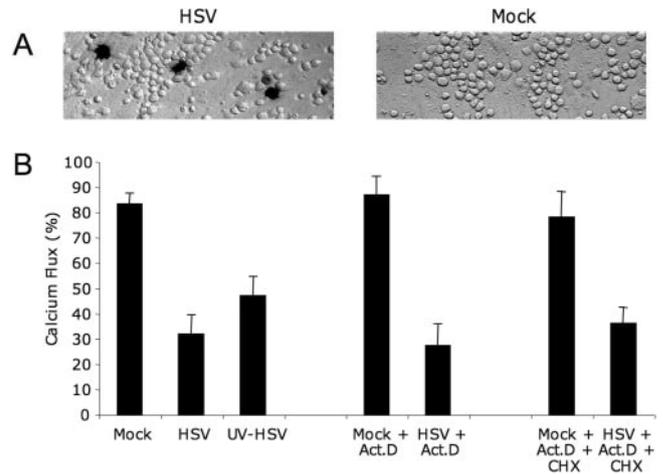
To assess the role of HSV entry in T cell inactivation, we infected Jurkat T cells with a HSV construct that expresses GFP upon cellular entry (26). GFP-HSV-infected T cells were triggered through the TCR and calcium flux was measured. Calcium flux analysis of a mixed population of GFP-expressing T cells demonstrated that inhibition of calcium mobilization was invariably associated with expression of GFP (Fig. 3A). The percentage of GFP-positive cells infected with GFP-HSV increased as the number of HSV particles per T cell (denoted by MOI) increased. Over a broad MOI range, there was a strong inverse relationship between GFP expression in T cells and the ability to flux calcium (Fig. 3B).

To further establish the requirement for HSV penetration, we assessed how an inhibitor of HSV fusion affected T cell inactivation. It was previously reported that a peptide denoted EB inhibited HSV entry into cells (33, 40). The EB peptide consists of an altered membrane-transiting motif derived from the FGF4 signal sequence. Although the exact mechanism of EB peptide action is not known, it has been shown to inhibit the fusion of preadsorbed HSV into cells without decreasing cellular or viral viability. In these experiments, HSV was preadsorbed to T cells on ice. The EB peptide or an irrelevant peptide was then added, and the cells were warmed to allow the energy-dependent process of membrane fusion to occur (30). At the maximal concentrations of EB peptide tested, there was no reduction in T cell viability (our unpublished observation). Furthermore, we detected no change in calcium flux in T cells incubated with the EB peptide alone, suggesting that the EB peptide did not interfere with TCR signaling (Fig. 3C). However, the EB peptide effectively rescued the calcium flux response in T cells inactivated by direct infection with preadsorbed HSV (Fig. 3C). With increasing concentrations of EB peptide tested, there was a dose-dependent decrease in HSV entry (decreased GFP expression) into T cells and a dose-dependent decrease in inactivation (Fig. 3D). Treatment with an irrelevant peptide did not inhibit GFP expression or inactivation (our unpublished observations). These results confirm that HSV adsorption is insufficient to inactivate T cells and that HSV penetration is required.

### Inactivation of T cells by HSV does not require transcription or translation

We have previously reported that HSV-infected fibroblasts inactivated T cells in the presence of acyclovir, an inhibitor of DNA synthesis. HSV DNA could not be detected in T cells inactivated in this manner (22). To examine whether HSV protein could be detected in T cells inactivated by acyclovir-treated, HSV-infected fibroblasts, we used the HSV reporter-plasmid, pMLP01. pMLP01 expresses  $\beta$ -galactosidase under control of the HSV promoter for ICP4 (29). After penetration and delivery of the HSV tegument protein VP16, cells transfected with pMLP01 can be visualized microscopically, as they turn blue in the presence of X-gal. We were able to detect VP16 in pMLP01-transfected Jurkat T cells incubated on HSV-infected fibroblasts in the presence of acyclovir. However, we were unable to detect VP16 in pMLP01-transfected Jurkat T cells incubated with mock-infected fibroblasts (Fig. 4A).

We had previously reported that treatment of T cells with an inhibitor of protein synthesis, cycloheximide (CHX), did not prevent inactivation by HSV-infected fibroblasts (22). In this report, we have demonstrated that HSV protein can transfer to the T cell from HSV-infected fibroblasts in the presence of acyclovir and that direct infection of T cells with HSV leads to inactivation. To investigate the role of the HSV virion in inactivation, we treated Jurkat T cells with UV-treated HSV, or we infected T cells with HSV in the presence of CHX and actinomycin D (ActD), an in-



**FIGURE 4.** HSV protein can be detected in Jurkat T cells inactivated with HSV-infected fibroblasts, but inhibition of TCR signal transduction does not require de novo transcription or translation. *A*, Jurkat T cells were transiently transfected with a HSV reporter construct expressing the  $\beta$ -galactosidase gene driven by a HSV promoter, incubated on HSV-1-infected or mock-infected fibroblasts as before, fixed, and stained with X-gal to visualize the presence of HSV protein in T cells. *B*, Jurkat T cells were mock-infected, infected with HSV-1F at an MOI of 100, or treated with 100 UV-treated HSV particles per cell. Alternatively, Jurkat T cells were treated for 1 h with ActD at 0.2  $\mu$ g/ml or ActD plus CHX at 10  $\mu$ g/ml and infected with HSV-1 at a MOI of 250 for 6 h. Results represent the average of three independent trials.

hibitor of de novo mRNA synthesis. In ActD- and CHX-treated T cells and with UV-HSV, we observed reduced calcium flux following TCR stimulation (Fig. 4B). In all of these conditions, HSV gene expression was effectively inhibited as measured by reduced GFP expression using a GFP-expressing HSV construct (our unpublished observations). Taken together, these results suggest that the HSV virion is sufficient to inhibit TCR signal transduction.

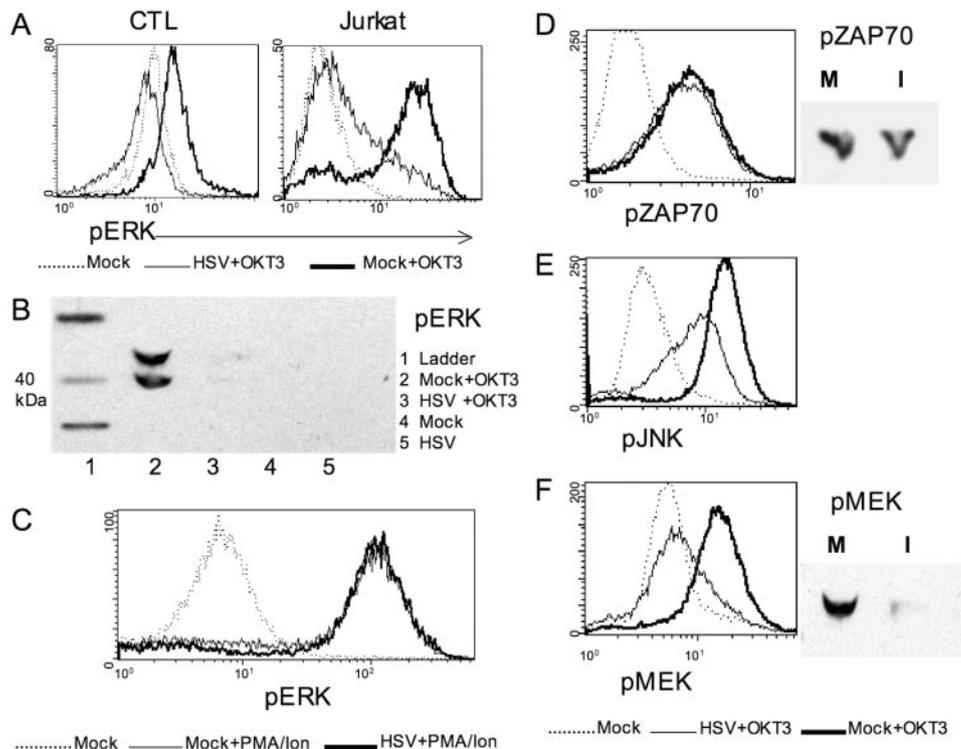
### ERK is not phosphorylated in HSV-inactivated T cells stimulated through the TCR

Stimulation of T cells through the TCR triggers an intracellular signaling cascade that, among other changes, leads to calcium mobilization and increased phosphorylation of MAPK. We have demonstrated defects in effector functions and in the ability to flux calcium in T cells inactivated by HSV. To determine whether HSV-inactivated T cells have additional defects in TCR signal transduction, we examined whether ERK, a central, downstream player in the TCR signaling machinery, is tyrosine phosphorylated in HSV-inactivated T cells triggered through the TCR. Using a phosphospecific Ab that recognizes the activated, tyrosine-phosphorylated form of ERK (pERK), we analyzed pERK levels in mock and HSV-inactivated T cells. pERK levels in CTL and Jurkat T cells inactivated on HSV-infected fibroblasts were dramatically reduced following TCR triggering as measured by flow cytometry and by immunoblotting (Fig. 5, *A* and *B*). These results suggest that tyrosine phosphorylation of a prominent MAPK is blocked in HSV-inactivated T cells.

### ERK is phosphorylated in HSV-inactivated T cells stimulated with PMA and ionomycin

Treatment of T cells with the mitogen PMA plus the calcium ionophore ionomycin has been shown to bypass proximal TCR signaling machinery and maximally activate T cells (41). Unlike TCR stimulation, stimulation with PMA and ionomycin increased ERK

**FIGURE 5.** Zap70 phosphorylation is normal in inactivated Jurkat T cells, but phosphorylation of MEK, ERK, and JNK is inhibited. *A*, Primary CTL and (*A–F*) Jurkat T cells were incubated on mock-infected fibroblasts or inactivated on HSV-2-infected fibroblasts. *A* and *B*, *D–F*, Mock and inactivated T cells were TCR stimulated with OKT3 or (*C*) stimulated with PMA plus ionomycin. Cells were fixed, stained, and analyzed by flow cytometry. Alternatively, cells were lysed, run on 4–12% acrylamide gels, transferred to nitrocellulose, and immunoblotted. *A–C*, Levels of phosphorylated ERK, (*D*) Zap70, (*E*) JNK, and (*F*) MEK were measured using phosphospecific Abs. M, mock. I, inactivated.



phosphorylation in both mock-treated T cells and HSV-inactivated T cells (Fig. 5C).

#### *In inactivated T cells, TCR signal transduction is inhibited distal to Zap70*

To delineate where the blockade in TCR signaling occurs in T cells inactivated by HSV, we investigated signaling events proximal to calcium mobilization and phosphorylation of ERK. The earliest events in TCR signaling involve tyrosine phosphorylation of the TCR  $\zeta$ -chain and CD3 by Lck and Fyn, members of the Src family kinases (42). The activated TCR-CD3 receptor complex recruits and phosphorylates Zap70. Using a phosphospecific Ab against Zap70, we determined that tyrosine phosphorylation of Zap70 occurred normally in HSV-inactivated T cells following TCR-CD3 ligation (Fig. 5D). In contrast, tyrosine phosphorylation of MAPK distal to Zap70, e.g., JNK and MEK, were inhibited in HSV-inactivated T cells stimulated through the TCR (Fig. 5, *E* and *F*).

#### *Tyrosine phosphorylation of LAT is reduced in HSV-inactivated T cells*

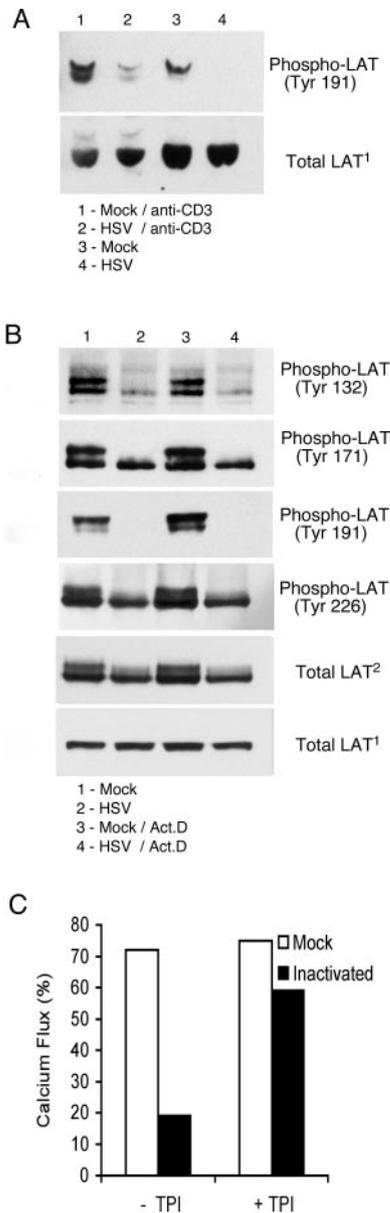
The phosphorylated forms of Zap70 and Lck function together to phosphorylate LAT at multiple tyrosine residues, a step that is necessary for TCR signal propagation. Phosphorylation of three of these critical residues (Tyr<sup>132</sup>, Tyr<sup>171</sup>, and Tyr<sup>191</sup>) are necessary and sufficient to mobilize calcium following TCR stimulation, while complete activation of ERK requires phosphorylation of Tyr<sup>110</sup> and Tyr<sup>226</sup> (43). LAT is expressed as a 42-kDa form (denoted here as LAT<sup>1</sup>) that results from alternative splicing and as 36- and 38-kDa forms (denoted here as LAT<sup>2</sup>) that result from posttranslational modifications (44). The two smaller forms of LAT are detected by phospho-LAT-specific Abs when T cells are stimulated. We determined that HSV-inactivated Jurkat T cells stimulated through the TCR failed to phosphorylate LAT at Tyr<sup>191</sup>. This was seen as a reduction in both the 36- and 38-kDa bands (Fig. 6A). The basal phosphorylation level of LAT at Tyr<sup>191</sup> was actually reduced before TCR stimulation (Fig. 6A). Levels of total

LAT were unchanged in the 36-kDa and the 42-kDa forms. There was a slight reduction in the 38-kDa form of total LAT in HSV-inactivated Jurkat T cells (Fig. 6B). We next investigated whether inactivation by HSV reduced the phosphorylation of LAT at additional tyrosine residues involved in TCR signaling (Fig. 6B). As seen with Tyr<sup>191</sup>, HSV reduced the phosphorylation of LAT at Tyr<sup>132</sup> in both the 36- and 38-kDa bands. In contrast, HSV reduced the phosphorylation of Tyr<sup>171</sup> and at Tyr<sup>226</sup> at the 38-kDa form of LAT but not at the 36-kDa form. The inclusion of ActD did not prevent the observed reductions in LAT phosphorylation, suggesting that *de novo* transcription in the T cell was not required (Fig. 6B). To determine whether T cell inactivation is dependent on the decrease in LAT phosphorylation, we treated T cells with tyrosine phosphatase inhibitors (TPI) before and during HSV infection. T cells treated with TPI were not inactivated as well as untreated T cells, further implicating the role of decreased LAT phosphorylation in HSV-induced inactivation (Fig. 6C).

## Discussion

We have shown that T cells inactivated by HSV or HSV-infected fibroblasts have profound inhibition in multiple TCR signaling intermediates as well as multiple downstream defects in effector functions. The reduced levels of tyrosine-phosphorylated LAT, which is critical for TCR signal transduction, may provide a mechanistic explanation for this observed phenotype. Entry of virus into T cells or transfer of a factor from HSV-infected cells is required for this inactivation, but within the T cell, viral replication and viral gene expression are not required. The simplest interpretation of these results is that an HSV virion component is responsible for the reduced levels of phosphorylated LAT. TPI were shown to restore T cell activation, suggesting that the inhibition of TCR signaling by HSV may result from a viral phosphatase or from activation or recruitment of a cellular phosphatase.

One example of modulation of cellular phosphorylation by HSV is the ICP34.5 protein. ICP34.5 is a virion protein that inhibits the



**FIGURE 6.** Tyrosine phosphorylation of LAT is reduced in HSV-inactivated T cells. *A*, Jurkat T cells were incubated on mock-infected fibroblasts or inactivated on HSV-2-infected fibroblasts and triggered with OKT3. *B*, Jurkat T cells were inactivated in the presence or absence of ActD without OKT3 triggering. *A* and *B*, T cells were lysed, run on 4–12% denaturing gel, transferred to nitrocellulose, and blotted with Abs that recognize specific forms of LAT tyrosine-phosphorylated at amino acid residues 191, 132, 171, and 226. Alternatively, blots were probed with mAbs against the three forms of total LAT (LAT<sup>1</sup> recognizes a single band at 42 kDa, LAT<sup>2</sup> recognizes two bands at 36 and 38 kDa). Each blot is representative of at least two experiments for each Ab. *C*, Jurkat T cells were untreated or treated for 1 h with a mixture of TPI. TPI was maintained throughout the assay. Cells were mock-infected or infected with HSV-1F at an MOI of 125 for 4 h in the presence of acyclovir, and calcium flux was determined as described previously. The results are representative of two independent experiments each.

action of dsRNA-dependent protein kinase R by activating the cellular serine-threonine protein phosphatase 1 (45, 46). Others have proposed that cellular protein tyrosine phosphatases, such as CTLA-4 and CD148, are excellent candidates to regulate TCR signaling, as they have been shown to inhibit proximal TCR signal transduction by inhibiting phosphorylation of Zap70 (47) and LAT

(18), respectively, in T cells. Although no known HSV proteins have been reported to inhibit TCR signaling, the tyrosine kinase-interacting protein of *Herpesvirus saimiri* has been shown to inhibit TCR signaling by inhibiting Zap70 phosphorylation (48).

Zap70 phosphorylation was intact in HSV-inactivated T cells stimulated through the TCR. However, the next step in TCR signaling, tyrosine phosphorylation of LAT, did not occur normally. The essential role of LAT in TCR signaling has been demonstrated in LAT-deficient Jurkat T cell mutants (20). When stimulated through the TCR, signaling in LAT-deficient mutants does not proceed to downstream events such as calcium flux and ERK phosphorylation. Before TCR stimulation, we observed that HSV-inactivated T cells had decreased basal levels of LAT phosphorylated at Tyr<sup>132</sup> and Tyr<sup>191</sup>, residues necessary for calcium mobilization in Jurkat T cells stimulated through the TCR (43). The interpretation of phosphorylation of LAT at Tyr<sup>171</sup> and Tyr<sup>226</sup> is more complex, as total LAT levels were also reduced slightly at the 38-kDa form. It is possible that HSV-induced inactivation sequesters or degrades this form of total LAT. Alternatively, it may be that the reduction in phosphorylation of this form of LAT leads indirectly to decreased stability. The reduction in LAT phosphorylation may result from decreased kinase activity or increased phosphatase activity in HSV-inactivated T cells. Reduced phosphorylation of LAT would provide a plausible explanation for the TCR-signaling block detected at the level of LAT. Two findings strengthened this reasoning. First, TPIs reversed inactivation. Second, the inactivating block at LAT was bypassed by stimulation with PMA plus ionomycin. PMA activates protein kinase C and ionomycin mobilizes calcium leading to the activation of ERK (49, 50), steps that are distal to the phosphorylation of LAT in TCR signal transduction.

HSV can infect primary T cells and Jurkat T cells (51, 52), and HSV infection may induce apoptosis or lead to lytic destruction of the T cell (53). HSV-induced lysis or apoptosis might explain the observations made by several groups, wherein contact with HSV-infected fibroblasts diminished the cytotoxic capacity of various immune cells (38, 54–57). The molecular mechanisms of inhibition of immune cell function were not determined in these reports. We have previously reported that T cells inactivated by HSV-infected fibroblasts in the presence of acyclovir were not productively infected nor apoptotic (22). Subsequent to that report, we determined that serum from HSV seropositive patients diminished the ability of HSV-infected cells to inactivate T cells, while serum from HSV seronegative patients had no effect. In addition, cells from several species infected with HSV could inactivate T cells (our unpublished observations). At that time, we considered the possibility that a viral protein expressed on the surface of HSV-infected cells might be responsible for inactivating T cells. To evaluate this hypothesis, we began looking for HSV glycoproteins that were required for inactivation. Fibroblasts infected with a HSV mutant deleted for gJ or gC, glycoproteins not involved in HSV entry, were able to inactivate T cells. In contrast, deletions in gB, gD, gH, gL, gE, or gI abolished inactivation by HSV-infected fibroblasts. Each of these glycoproteins is involved in viral fusion or viral cell-to-cell spread. Given our findings that multiple glycoproteins involved in HSV entry are required to inactivate T cells, it seems unlikely that ligation of multiple cellular receptors inhibits TCR signaling in the absence of HSV entry.

HSV gD binds modified forms of heparan sulfate, nectin-1, nectin-2, and HVEM (58). HVEM has two known cellular ligands LIGHT and B and T lymphocyte attenuator, each reportedly found on T cells. Although binding of HVEM to LIGHT can send a costimulatory signal to T cells (59), binding of HVEM to B and T lymphocyte attenuator can send a coinhibitory signal to T cells

(60). Therefore, it was important to determine whether HVEM ligation was involved in the inactivation of T cells by HSV. We established that HSV gD mutants that could not bind HVEM could inactivate T cells, suggesting that HVEM is not directly mediating inactivation.

We have reported that the HSV virion is sufficient to inactivate T cells. HSV treated with UV light was capable of inactivating T cells when added directly to the T cells. UV treatment induces thymidine dimers in DNA that inhibits DNA replication and transcription. The UV dosage used did not prevent HSV cell penetration (our unpublished observation). To support the UV-HSV experiment, we demonstrated that inhibitors of transcription and translation (ActD and CHX, respectively) did not prevent inactivation. It should be noted that relatively high numbers of virion per T cell were required to cause inactivation with UV-treated HSV or with HSV infection in the presence of ActD and CHX. This is likely because the only protein available to elicit TCR signal inhibition had to be provided in the virion, as HSV protein would not accumulate during inhibition of de novo transcription and translation.

Although inactivation requires HSV entry into T cells, viral gene expression and viral replication are not required. Acyclovir inhibits DNA synthesis in HSV-infected cells, but acyclovir does not inhibit inactivation of T cells by HSV or by HSV-infected fibroblasts. From our previous report using real-time PCR, HSV DNA could not be detected in T cells inactivated on acyclovir-treated, HSV-infected fibroblasts (22). In this report, we were able to detect HSV protein in T cells inactivated on acyclovir-treated, HSV-infected fibroblasts. It is possible that HSV protein transfers from HSV-infected cells to T cells without transferring viral DNA. HSV-infected cells can make significant numbers of replication-defective particles that lack viral DNA and capsids (61, 62). In the presence of acyclovir, a similar defective HSV particle, the pre-viral replication enveloped particle, can reportedly deliver HSV proteins into cells (63). If we can confirm that purified HSV replication-defective particles directly inactivate T cells, this may be of considerable interest to researchers studying these particles as vaccine candidates and as gene therapy vectors (64, 65). In addition, this work may implicate replication-defective particles as a source of immune suppression in HSV reactivation during acyclovir therapy.

As an alternative explanation for our findings, HSV proteins may be able to spread from infected cells to uninfected cells via plasma membrane protrusions, a process that may not involve production of viral particles (66). It is also possible that another virion component, such as mRNA, could mediate inactivation, although we consider protein(s) to be more likely candidates. Finally, it is possible that the fusion process alone may be sufficient for inactivation. Penetration of HSV has been associated with tyrosine phosphorylation of cellular proteins (67) and with activation of calcium-signaling pathways (68). However, there have been no previous reports of TCR signal inhibition or reduced tyrosine phosphorylation associated with HSV fusion or entry.

In this report, we have outlined a novel mechanism of viral immune evasion, whereby HSV virion protein profoundly inhibits TCR signaling. Inactivation involves reduced phosphorylation of LAT at tyrosine residues that are critical for TCR signal transduction, providing one possible mechanism for the inhibition of T cell effector function by HSV. A complex relationship exists between T cells and HSV. Although T cells are needed to control HSV infection, they do not eradicate HSV or prevent reactivation. The direct effect that HSV has on the T cell is not well understood, and it may be that inactivation gives HSV an advantage in the establishment of latency or during reactivation. At this time, there is no

evidence that HSV inhibits T cell function in vivo. It seems unlikely that HSV systemically inactivates T cells, given that the high prevalence rate of HSV-1 does not lead to a generalized immunocompromised state. A more likely scenario for the in vivo relevance of HSV-induced inactivation is a localized effect that stuns T cells long enough to allow HSV to establish latent infection. Regardless of the possible in vivo relevance of inactivation in HSV pathogenesis, a mechanism to specifically inhibit TCR signaling could be of benefit in a variety of diseases involving insufficient or excessive T cell function, e.g., tumor surveillance (69), transplant rejection (70), and graft-vs-host-disease (71). Understanding how HSV inactivates T cells may provide insight to better manage HSV infection. In a broader sense, the ability to directly inhibit TCR signal transduction by dephosphorylating LAT may ultimately allow targeted manipulation of T cell function.

## Disclosures

The authors have no financial conflict of interest.

## References

- Koelle, D. M., C. M. Posavad, G. R. Barnum, M. L. Johnson, J. M. Frank, and L. Corey. 1998. Clearance of HSV-2 from recurrent genital lesions correlates with infiltration of HSV-specific cytotoxic T lymphocytes. *J. Clin. Invest.* 101: 1500–1508.
- Posavad, C. M., D. M. Koelle, and L. Corey. 1996. High frequency of CD8<sup>+</sup> cytotoxic T-lymphocyte precursors specific for herpes simplex viruses in persons with genital herpes. *J. Virol.* 70: 8165–8168.
- Bonneau, R. H., and S. R. Jennings. 1989. Modulation of acute and latent herpes simplex virus infection in C57BL/6 mice by adoptive transfer of immune lymphocytes with cytolytic activity. *J. Virol.* 63: 1480–1484.
- Johnson, R. E., A. J. Nahmias, L. S. Magder, F. K. Lee, C. A. Brooks, and C. B. Snowden. 1989. A seroepidemiologic survey of the prevalence of herpes simplex virus type 2 infection in the United States. *N. Engl. J. Med.* 321: 7–12.
- Higgins, C. R., J. K. Schofield, F. M. Tatnall, and I. M. Leigh. 1993. Natural history, management and complications of herpes labialis. *J. Med. Virol.* (Suppl. 1): 22–26.
- Friedman, E., A. H. Katcher, and V. J. Brightman. 1977. Incidence of recurrent herpes labialis and upper respiratory infection: a prospective study of the influence of biologic, social, and psychological predictors. *Oral Surg.* 43: 873–878.
- Streilein, J. W., M. R. Dana, and B. R. Ksander. 1997. Immunity causing blindness: five different paths to herpes stromal keratitis. *Immunol. Today* 18: 443–449.
- Olson, L. C., E. L. Buescher, M. S. Artenstein, and P. D. Parkman. 1967. Herpesvirus infections of the human central nervous system. *N. Engl. J. Med.* 277: 1271–1277.
- Quinnan, G. V., Jr., H. Masur, A. H. Rook, G. Armstrong, W. R. Frederick, J. Epstein, J. F. Manischewitz, A. M. Macher, L. Jackson, J. Ames, et al. 1984. Herpesvirus infections in the acquired immune deficiency syndrome. *J. Am. Med. Assoc.* 252: 72–77.
- Wald, A., T. Schacker, and L. Corey. 1997. HSV-2 and HIV: consequences of an endemic opportunistic infection. *STEP Perspect.* 9: 2–4.
- Posavad, C. M., D. M. Koelle, and L. Corey. 1998. Tipping the scales of herpes simplex virus reactivation: the important responses are local. *Nat. Med.* 4: 381–382.
- Hill, A., P. Jugovic, I. York, G. Russ, J. Bennink, J. Yewdell, H. Ploegh, and D. Johnson. 1995. Herpes simplex virus turns off the TAP to evade host immunity. *Nature* 375: 411–415.
- Koelle, D. M., M. A. Tigges, R. L. Burke, F. W. Symington, S. R. Riddell, H. Abbo, and L. Corey. 1993. Herpes simplex virus infection of human fibroblasts and keratinocytes inhibits recognition by cloned CD8<sup>+</sup> cytotoxic T lymphocytes. *J. Clin. Invest.* 91: 961–968.
- York, I. A., C. Roop, D. W. Andrews, S. R. Riddell, F. L. Graham, and D. C. Johnson. 1994. A cytosolic herpes simplex virus protein inhibits antigen presentation to CD8<sup>+</sup> T lymphocytes. *Cell* 77: 525–535.
- Jerome, K. R., Z. Chen, R. Lang, M. R. Torres, J. Hofmeister, S. Smith, R. Fox, C. J. Froelich, and L. Corey. 2001. HSV and glycoprotein J inhibit caspase activation and apoptosis induced by granzyme B or Fas. *J. Immunol.* 167: 3928–3935.
- Jerome, K. R., R. Fox, Z. Chen, A. E. Sears, H. Lee, and L. Corey. 1999. Herpes simplex virus inhibits apoptosis through the action of two genes, Us5 and Us3. *J. Virol.* 73: 8950–8957.
- Lin, J., and A. Weiss. 2001. T cell receptor signalling. *J. Cell Sci.* 114: 243–244.
- Baker, J. E., R. Majeti, S. G. Tangye, and A. Weiss. 2001. Protein tyrosine phosphatase CD148-mediated inhibition of T-cell receptor signal transduction is associated with reduced LAT and phospholipase C $\gamma$ 1 phosphorylation. *Mol. Cell. Biol.* 21: 2393–2403.
- Williams, B. L., K. L. Schreiber, W. Zhang, R. L. Wange, L. E. Samelson, P. J. Leibson, and R. T. Abraham. 1998. Genetic evidence for differential coupling of Syk family kinases to the T-cell receptor: reconstitution studies in a ZAP-70-deficient Jurkat T-cell line. *Mol. Cell. Biol.* 18: 1388–1399.

20. Zhang, W., B. J. Irvin, R. P. Tribble, R. T. Abraham, and L. E. Samelson. 1999. Functional analysis of LAT in TCR-mediated signaling pathways using a LAT-deficient Jurkat cell line. *Int. Immunol.* 11: 943–950.
21. Mustelin, T., A. Alonso, N. Bottini, H. Huynh, S. Rahmouni, K. Nika, C. Louis-dit-Sully, L. Tautz, S. H. Togo, S. Bruckner, et al. 2004. Protein tyrosine phosphatases in T cell physiology. *Mol. Immunol.* 41: 687–700.
22. Sloan, D. D., G. Zahariadis, C. M. Posavad, N. T. Pate, S. J. Kussick, and K. R. Jerome. 2003. CTL are inactivated by herpes simplex virus-infected cells expressing a viral protein kinase. *J. Immunol.* 171: 6733–6741.
23. Brodie, S. J., D. A. Lewinsohn, B. K. Patterson, D. Jiyamapa, J. Krieger, L. Corey, P. D. Greenberg, and S. R. Riddell. 1999. In vivo migration and function of transferred HIV-1-specific cytotoxic T cells. *Nat. Med.* 5: 34–41.
24. Rauch, D. A., N. Rodriguez, and R. J. Roller. 2000. Mutations in herpes simplex virus glycoprotein D distinguish entry of free virus from cell-cell spread. *J. Virol.* 74: 11437–11446.
25. Forrester, A., H. Farrell, G. Wilkinson, J. Kaye, N. Davis-Poynter, and T. Minson. 1992. Construction and properties of a mutant of herpes simplex virus type 1 with glycoprotein H coding sequences deleted. *J. Virol.* 66: 341–348.
26. Farnsworth, A., K. Goldsmith, and D. C. Johnson. 2003. Herpes simplex virus glycoproteins gD and gE/gI serve essential but redundant functions during acquisition of the virion envelope in the cytoplasm. *J. Virol.* 77: 8481–8494.
27. Ligas, M. W., and D. C. Johnson. 1988. A herpes simplex virus mutant in which glycoprotein D sequences are replaced by  $\beta$ -galactosidase sequences binds to but is unable to penetrate into cells. *J. Virol.* 62: 1486–1494.
28. Cai, W. Z., S. Person, S. C. Warner, J. H. Zhou, and N. A. DeLuca. 1987. Linker-insertion nonsense and restriction-site deletion mutations of the gB glycoprotein gene of herpes simplex virus type 1. *J. Virol.* 61: 714–721.
29. Montgomery, R. I., M. S. Warner, B. J. Lum, and P. G. Spear. 1996. Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. *Cell* 87: 427–436.
30. Herold, B. C., R. J. Visalli, N. Susmarski, C. R. Brandt, and P. G. Spear. 1994. Glycoprotein C-independent binding of herpes simplex virus to cells requires cell surface heparan sulphate and glycoprotein B. *J. Gen. Virol.* 75(Pt. 6): 1211–1222.
31. Yoon, M., A. Zago, D. Shukla, and P. G. Spear. 2003. Mutations in the N termini of herpes simplex virus type 1 and 2 gDs alter functional interactions with the entry/fusion receptors HVEM, nectin-2, and 3-O-sulfated heparan sulfate but not with nectin-1. *J. Virol.* 77: 9221–9231.
32. Yoon, M., and P. G. Spear. 2004. Random mutagenesis of the gene encoding a viral ligand for multiple cell entry receptors to obtain viral mutants altered for receptor usage. *Proc. Natl. Acad. Sci. USA* 101: 17252–17257.
33. Bultmann, H., J. S. Busse, and C. R. Brandt. 2001. Modified FGF4 signal peptide inhibits entry of herpes simplex virus type 1. *J. Virol.* 75: 2634–2645.
34. Abraham, R. T. 2000. Mutant T cell lines as model systems for the dissection of T cell antigen receptor signaling pathways. *Immunol. Res.* 22: 95–117.
35. Tsoukas, C. D., B. Landgraf, J. Bentin, M. Valentine, M. Lotz, J. H. Vaughan, and D. A. Carson. 1985. Activation of resting T lymphocytes by anti-CD3 (T3) antibodies in the absence of monocytes. *J. Immunol.* 135: 1719–1723.
36. McMurray, R. W., K. Ndebele, K. J. Hardy, and J. K. Jenkins. 2001. 17- $\beta$ -estradiol suppresses IL-2 and IL-2 receptor. *Cytokine* 14: 324–333.
37. Dingwell, K. S., C. R. Brunetti, R. L. Hendricks, Q. Tang, M. Tang, A. J. Rainbow, and D. C. Johnson. 1994. Herpes simplex virus glycoproteins E and I facilitate cell-to-cell spread in vivo and across junctions of cultured cells. *J. Virol.* 68: 834–845.
38. York, I. A., and D. C. Johnson. 1993. Direct contact with herpes simplex virus-infected cells results in inhibition of lymphokine-activated killer cells because of cell-to-cell spread of virus. *J. Infect. Dis.* 168: 1127–1132.
39. Piret, J., J. Lamontagne, J. Bestman-Smith, S. Roy, P. Gourde, A. Desormeaux, R. F. Omar, J. Juhasz, and M. G. Bergeron. 2000. In vitro and in vivo evaluations of sodium lauryl sulfate and dextran sulfate as microbicides against herpes simplex and human immunodeficiency viruses. *J. Clin. Microbiol.* 38: 110–119.
40. Bultmann, H., and C. R. Brandt. 2002. Peptides containing membrane-transiting motifs inhibit virus entry. *J. Biol. Chem.* 277: 36018–36023.
41. Herndon, T. M., X. C. Shan, G. C. Tsokos, and R. L. Wange. 2001. ZAP-70 and SLP-76 regulate protein kinase C- $\theta$  and NF- $\kappa$ B activation in response to engagement of CD3 and CD28. *J. Immunol.* 166: 5654–5664.
42. Iwashima, M., B. A. Irving, N. S. van Oers, A. C. Chan, and A. Weiss. 1994. Sequential interactions of the TCR with two distinct cytoplasmic tyrosine kinases. *Science* 263: 1136–1139.
43. Lin, J., and A. Weiss. 2001. Identification of the minimal tyrosine residues required for linker for activation of T cell function. *J. Biol. Chem.* 276: 29588–29595.
44. Zhang, W., J. Sloan-Lancaster, J. Kitchen, R. P. Tribble, and L. E. Samelson. 1998. LAT: the ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation. *Cell* 92: 83–92.
45. Mao, H., and K. S. Rosenthal. 2002. An N-terminal arginine-rich cluster and a proline-alanine-threonine repeat region determine the cellular localization of herpes simplex virus type 1 ICP34.5 protein and its ligand, protein phosphatase 1. *J. Biol. Chem.* 277: 11423–11431.
46. Harland, J., P. Dunn, E. Cameron, J. Conner, and S. M. Brown. 2003. The herpes simplex virus (HSV) protein ICP34.5 is a virion component that forms a DNA-binding complex with proliferating cell nuclear antigen and HSV replication proteins. *J. Neurovirol.* 9: 477–488.
47. Guntermann, C., and D. R. Alexander. 2002. CTLA-4 suppresses proximal TCR signaling in resting human CD4<sup>+</sup> T cells by inhibiting ZAP-70 Tyr<sup>319</sup> phosphorylation: a potential role for tyrosine phosphatases. *J. Immunol.* 168: 4420–4429.
48. Cho, N. H., P. Feng, S. H. Lee, B. S. Lee, X. Liang, H. Chang, and J. U. Jung. 2004. Inhibition of T cell receptor signal transduction by tyrosine kinase-interacting protein of *Herpesvirus saimiri*. *J. Exp. Med.* 200: 681–687.
49. Elzi, D. J., A. J. Bjornsen, T. MacKenzie, T. H. Wyman, and C. C. Silliman. 2001. Ionomycin causes activation of p38 and p42/44 mitogen-activated protein kinases in human neutrophils. *Am. J. Physiol.* 281: C350–C360.
50. Castagna, M., Y. Takai, K. Kaibuchi, K. Sano, U. Kikkawa, and Y. Nishizuka. 1982. Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J. Biol. Chem.* 257: 7847–7851.
51. Hammer, S. M., W. P. Carney, V. R. Iacoviello, B. R. Lowe, and M. S. Hirsch. 1982. Herpes simplex virus infection of human T-cell subpopulations. *Infect. Immun.* 38: 795–797.
52. Braun, R. W., H. K. Teute, H. Kirchner, and K. Munk. 1984. Replication of herpes simplex virus in human T lymphocytes: characterization of the viral target cell. *J. Immunol.* 132: 914–919.
53. Raftery, M. J., C. K. Behrens, A. Muller, P. H. Krammer, H. Walczak, and G. Schonrich. 1999. Herpes simplex virus type 1 infection of activated cytotoxic T cells: induction of fratricide as a mechanism of viral immune evasion. *J. Exp. Med.* 190: 1103–1114.
54. Confer, D. L., G. M. Vercellotti, D. Kotasek, J. L. Goodman, A. Ochoa, and H. S. Jacob. 1990. Herpes simplex virus-infected cells disarm killer lymphocytes. *Proc. Natl. Acad. Sci. USA* 87: 3609–3613.
55. Posavad, C. M., J. J. Newton, and K. L. Rosenthal. 1993. Inhibition of human CTL-mediated lysis by fibroblasts infected with herpes simplex virus. *J. Immunol.* 151: 4865–4873.
56. Posavad, C. M., J. J. Newton, and K. L. Rosenthal. 1994. Infection and inhibition of human cytotoxic T lymphocytes by herpes simplex virus. *J. Virol.* 68: 4072–4074.
57. Posavad, C. M., and K. L. Rosenthal. 1992. Herpes simplex virus-infected human fibroblasts are resistant to and inhibit cytotoxic T-lymphocyte activity. *J. Virol.* 66: 6264–6272.
58. Spear, P. G. 2004. Herpes simplex virus: receptors and ligands for cell entry. *Cell. Microbiol.* 6: 401–410.
59. Croft, M. 2003. Co-stimulatory members of the TNFR family: keys to effective T-cell immunity? *Nat. Rev. Immunol.* 3: 609–620.
60. Croft, M. 2005. The evolving crosstalk between co-stimulatory and co-inhibitory receptors: HVEM-BTLA. *Trends Immunol.* 26: 292–294.
61. Dargan, D. J., and J. H. Subak-Sharpe. 1997. The effect of herpes simplex virus type 1 L-particles on virus entry, replication, and the infectivity of naked herpesvirus DNA. *Virology* 239: 378–388.
62. Szilagyi, J. F., and C. Cunningham. 1991. Identification and characterization of a novel non-infectious herpes simplex virus-related particle. *J. Gen. Virol.* 72(Pt. 3): 661–668.
63. Dargan, D. J., A. H. Patel, and J. H. Subak-Sharpe. 1995. PREPs: herpes simplex virus type 1-specific particles produced by infected cells when viral DNA replication is blocked. *J. Virol.* 69: 4924–4932.
64. Subak-Sharpe, J. H., and D. J. Dargan. 1998. HSV molecular biology: general aspects of herpes simplex virus molecular biology. *Virus Genes* 16: 239–251.
65. Pardoe, I., and D. Dargan. 2002. PREPS and L-particles: a new approach to virus-like particle vaccines. *Expert Rev. Vaccines* 1: 427–432.
66. La Boissiere, S., A. Izeta, S. Malcomber, and P. O'Hare. 2004. Compartmentalization of VP16 in cells infected with recombinant herpes simplex virus expressing VP16-green fluorescent protein fusion proteins. *J. Virol.* 78: 8002–8014.
67. Qie, L., D. Marcellino, and B. C. Herold. 1999. Herpes simplex virus entry is associated with tyrosine phosphorylation of cellular proteins. *Virology* 256: 220–227.
68. Cheshenko, N., B. Del Rosario, C. Woda, D. Marcellino, L. M. Satlin, and B. C. Herold. 2003. Herpes simplex virus triggers activation of calcium-signaling pathways. *J. Cell Biol.* 163: 283–293.
69. Amrolia, P. J., S. D. Reid, L. Gao, B. Schultheis, G. Dotti, M. K. Brenner, J. V. Melo, J. M. Goldman, and H. J. Stauss. 2003. Allo-restricted cytotoxic T cells specific for human CD45 show potent antileukemic activity. *Blood* 101: 1007–1014.
70. Valujskikh, A., O. Lantz, S. Celli, P. Matzinger, and P. S. Heeger. 2002. Cross-primed CD8<sup>+</sup> T cells mediate graft rejection via a distinct effector pathway. *Nat. Immunol.* 3: 844–851.
71. Barry, M., and R. C. Bleackley. 2002. Cytotoxic T lymphocytes: all roads lead to death. *Nat. Rev. Immunol.* 2: 401–409.