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CTL Are Inactivated by Herpes Simplex Virus-Infected Cells Expressing a Viral Protein Kinase

Derek D. Sloan,*† George Zahariadis,‡ Christine M. Posavad,*‡ Nichlos T. Pate,‡ Steven J. Kussick,* and Keith R. Jerome‡*†

Numerous cell-to-cell signals tightly regulate CTL function. Human fibroblasts infected with HSV type 1 or 2 can generate such a signal and inactivate human CTL. Inactivated CTL lose their ability to release cytotoxic granules and synthesize cytokines when triggered through the TCR. Inactivation requires cell-to-cell contact between CTL and HSV-infected cells. However, inactivated CTL are not infected with HSV. The inactivation of CTL is sustainable, as CTL function remains impaired when the CTL are removed from the HSV-infected cells. IL-2 treatment does not alter inactivation, and the inactivated phenotype is not transferable between CTL, distinguishing this phenotype from traditional anergy and T regulatory cell models. CTL inactivated by HSV-infected cells are not apoptotic, and the inactivated state can be overcome by phorbol ester stimulation, suggesting that inactivated CTL are viable and that the signaling block is specific to the TCR. HSV-infected cells require the expression of U_{3}, a viral protein kinase, to transmit the inactivating signal. Elucidation of the molecular nature of this signaling pathway may allow targeted manipulation of CTL function. The Journal of Immunology, 2003, 171: 6733–6741.

Because CTL are involved in controlling the acute, lytic phase of most viral infections, including HIV, hepatitis B virus, and the herpes viruses (e.g., HSV, CMV, and EBV) (1–6). CTL are also thought to play a role in cancer surveillance, and CTL clones raised against tumor Ags have been used successfully in adoptive immunotherapy regimens (7–10). However, CTL can cause life-threatening illnesses such as transplant rejection, graft-vs-host disease, and autoimmunity (11–13). Thus, CTL are linked to both preventing and promoting pathogenesis, and the ability to specifically modulate CTL function is an attractive therapy concept.

CTL are a critical component of many successful immune responses, but they have the potential to injure the host. Therefore, their activity is closely regulated by multiple receptor-ligand interactions. Before they can function as effectors, CTL must be activated by APCs and triggered by target cells. Once activated, the immune system has mechanisms to control the CTL response and prevent the destruction of healthy host cells. Activated CTL can be depleted through Fas-mediated apoptosis, a process called activation-induced cell death. Alternatively, activated CTL can become anergic or differentiate into a functionally impaired, nonresponsive, or memory-like state (14–16).

Because CTL are involved in controlling the acute, lytic phase and persistent, latent phase of HSV infection, HSV has evolved numerous mechanisms to evade CTL as part of its survival strategy. HSV-infected cells can avoid CTL detection by interfering with TAP-mediated peptide loading onto MHC (17–19). Furthermore, several HSV genes have been shown to inhibit CTL-induced apoptosis of HSV-infected target cells (20–24). An alternative mechanism has been described, wherein HSV-infected cells were capable of inhibiting the lytic function of various immune effector cells (25–27). In these models, when effector cells were incubated with HSV-infected fibroblasts, they lost the ability to lyse subsequently added target cells. However, the significance of these initial findings was not appreciated, as subsequent efforts focused on the cell-to-cell spread of HSV to effector cells (28, 29). In addition, it was reported that HSV-infected CTL expressed increased levels of Fas ligand and thus could be silenced through fratricide-induced apoptosis (30).

In this study, we demonstrate that HSV-infected fibroblasts transmit a functionally inhibiting signal to CTL without infecting CTL or inducing apoptosis in CTL. We refer to this process as inactivating. The inactivating signal markedly decreases the cytotoxic and cytokine effector functions of CTL, and the inactivated phenotype is sustainable after CTL are removed from HSV-infected cells and treated with IL-2. Although inactivated CTL cannot be stimulated through the TCR, they are capable of secreting IFN-γ when treated with a phorbol ester plus ionomycin. Finally, we have shown that HSV-infected fibroblasts require the expression of a viral protein kinase, U_{3}, to transmit the inactivating signal to CTL. Our HSV-mediated inactivation model may elucidate a mechanism that would allow specific manipulation of CTL function.

Materials and Methods

Cell lines and viruses

The HLA-A*0201-restricted CD8⁺ CTL clone, SKH13, recognizes the HA-8 minor histocompatibility human Ag (mHA-8).³ The HLA-A*3-restricted CD8⁺CTL clone, KSN, recognizes the peptide RVWDLPGVLK. Both CTL clones were stimulated using a 14-day schedule, as previously

³ Abbreviations used in this paper: mHA, HA-8 minor histocompatibility human Ag; BFA, brefeldin A; CHX, cycloheximide; LCL, lymphoblastoid cell line; MHC, MHC class I-like molecule; MOI, multiplicity of infection; SEB, staphylococcal enterotoxin B.
described (31, 32). SKH13, KSN, and the EBV-immortalized lymphoblastoid B cell lines (LCL), CEPH-8240 (mHA-8 positive), SKH (mHA-8 negative), KSN (peptide positive), and GAO (peptide negative) were kindly provided by E. Warren at the Fred Hutchinson Cancer Research Center. LCLs were grown in RPMI 1640 supplemented with 4 mM HEPES, 3 mM l-glutamine, 10% FCS, 50 U/ml penicillin, and 50 μg/ml streptomycin. Fibroblasts were obtained from human foreskin samples and maintained in DMEM, 10% FCS, 50 U/ml penicillin, and 50 μg/ml streptomycin. Human fibroblasts were used in assays from passage 5 to 12. Vero cells were obtained from the American Type Culture Collection (Manassas, VA) 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 (provided by J. Blaho (Mt. Sinai School of Medicine, New York, NY), originally obtained from B. Roizman (University of Chicago, Chicago, IL) (33)) and HSV-2 strain Hg52 were used in the experiments, as indicated. The mutant viruses were deletion-rescue pairs derived from HSV-1 F. The U12, IPCP47, and rescue viruses, vBS/H9004 and vBS/H9251, were provided by J. Blaho and originally obtained from P. Schaffer (Harvard Medical School, Boston, MA) (34). The U12 deletion and rescue viruses, IPCP47a and IPCP47AR, were provided by D. Johnson (Oregon Health and Science University, Portland, OR) (29). The U3 deletion and rescue viruses, R7041 and R7306, were provided by B. Roizman (35). The U5 deletion and rescue viruses, RAS116 and RAS137, were provided by A. Sears (Tampa Bay Research Institute, St. Petersburg, FL) (22). Because the U4 (ICP4) rescue virus was not available, the U4 deletion virus d120 was compared with HSV-1 parent strain KOS 1.1. These viruses were provided by J. Smiley (University of Alberta, Edmonton, Alberta, Canada) and originally obtained from N. DeLuca (University of Pittsburgh, Pittsburgh, PA) (36). All viruses were grown on Vero cells and titrated using standard plaque assays.

### Cell-mediated cytotoxicity assays

Fibroblasts were grown to confluency in 96-well, flat-bottom plates and infected with HSV at a multiplicity of infection (MOI) of 10 or mock infected. In some assays, fibroblasts and CTL were pretreated with acyclovir or cycloheximide (CHX) for the times and concentrations indicated (acyclovir and CHX from Sigma-Aldrich, St. Louis, MO). After a 6-h infection of fibroblasts with HSV in DMEM with 2% FCS (medium), fibroblasts were washed with PBS. CTL were added in 100 μl medium/well and incubated for 4 h at 37°C and 5% CO₂. These CTL are restricted to MHC molecules not expressed on the inactivating fibroblasts, and thus the CTL do not recognize the fibroblasts (data not shown). HA-8⁺ and HA-8⁻ LCL target cells (targets) were labeled with 100–200 μCi of Na₂¹³⁵CICO₄ (Amersham Pharmacia Biotech, Piscataway, NJ) for 2–4 h at 37°C, washed, and counted. If a three-cell sandwich chromium release assay was performed, ¹³⁵Cr-labeled targets in 100 μl/well were added to the HSV-infected fibroblasts and CTL at various E:T ratios. Alternatively, HSV-inactivated CTL were gently aspirated from HSV-infected fibroblasts. Visual inspection confirmed that fibroblasts were not removed during aspiration. Removed CTL were counted and added to new wells containing ¹³⁵Cr-labeled targets. CTL were incubated with ¹³⁵Cr-labeled targets for 5–6 h at 37°C. A total of 40 μl of supernatant/well was removed, added to a Lumaplate, and counted in a scintillation counter (Packard, Meriden, CT). Spontaneous and maximal release were measured by adding medium or 5% IGEPAL (Sigma-Aldrich) to ¹³⁵Cr-labeled targets. The spontaneous release was always less than 20% of the maximal release. All wells were done in triplicate, and percentage of specific ¹³⁵Cr release (%SR) was calculated by the formula %SR = ([mean experimental cpm – mean spontaneous cpm]/[mean maximal cpm – mean spontaneous cpm]) × 100. Percentage of inactivation = ((%SR from control – %SR from inactivated)/(%SR from control)) × 100, where the control group was CTL incubated on mock-infected fibroblasts and the inactivated group was CTL incubated on HSV-infected fibroblasts.

### Redirected cell lysis

CTL were incubated with mock- or HSV-infected fibroblasts and removed, as described above. CTL were then incubated with a mAb raised against CD3 (clone OKT3; Ortho-McNeil, Redwood City, CA) at 1 μg/ml for 15 min at 37°C. P815 cells overexpressing FcγR were kindly provided by V. Groh and T. Spies (Fred Hutchinson Cancer Research Center, Seattle, WA) (37). P815 cells were labeled with 100–200 μCi of Na₂¹³⁵CICO₄ for 2 h at 37°C, washed, counted, and then added to OKT3-bound CTL at various E:T ratios for 6 h.

### Flow cytometric analysis of granzyme A and cytokine production

CTL were incubated with mock- or HSV-infected fibroblasts and removed. Lytic granule content in CTL was determined by incubating CTL with LCL at an E:T ratio of 1:5 for 5 h. Cells were then stained with PerCP-conjugated anti-CD8 (clone SK1; BD PharMingen, San Diego, CA) and FITC-conjugated anti-granzyme A Ab using the granzyme A reagent kit (clone CB9; BD PharMingen) and analyzed by flow cytometry.

### Flow cytometric analysis for T cell signaling and activation-associated cell surface Ags and ligands

CTL were incubated with mock- or HSV-infected fibroblasts, and previously described, removed, and added to unlabeled LCL target cells at various E:T ratios for 6 h at 37°C. In some experiments, CTL were untreated, treated with 1 μg/ml staphylococcal enterotoxin B (SEB), or treated with 100 ng/ml PMA plus 1 μg/ml ionomycin for 6 h at 37°C (SEB, PMA, and ionomycin; Sigma-Aldrich). During the final 4 h of stimulation with BLCI or mitogens, 10 μg/ml brefeldin A (BFA; Sigma-Aldrich) was added to prevent protein secretion and allow visualization of the retained cytokines. CTL were fixed, permeabilized, and stained with PerCP-conjugated anti-CD8 (clone SK1), FITC-conjugated anti-IFN-γ (clone 25723.11), or FITC-conjugated anti-TNF-α (clone 6401.1111), following the manufacturer’s suggested protocol (BD PharMingen), and analyzed by flow cytometry.

### Flow cytometric analysis for T cell apoptosis

### Flow cytometric analysis of apoptotic markers

CTL were incubated with mock- or HSV-infected fibroblasts, and removed, and added to LCL. To distinguish BLCL from CTL, LCL were labeled with an integral membrane dye using the PKH26 Red Fluorescent Cell Link Kit (Sigma-Aldrich). CTL and PKH26-labeled LCL were incubated at various E:T ratios for 5 h, and activated caspase 3 was measured using the active caspase 3 FITC mAb Apopsis Kit 1 (clone C92-605; BD PharMingen). Cells were then paraformaldehyde fixed and analyzed by flow cytometry (40).

Mitochondrial potential was assessed in untreated CTL. CTL treated with 1 μM staurosporine (Sigma-Aldrich) for 4 h. CTL incubated with uninfected fibroblasts (control) for 4 h, and CTL incubated with HSV-infected fibroblasts (inactivated) for 4 h. CTL were then stained using MitoTracker Red CMXRs as per the manufacturer’s instructions (Molecular Probes, Eugene, OR). Control and inactivated CTL were also stained with Ab against CD95/Fas (clone DX2; DAKO, Carpenteria, CA) and with the DNA-binding dye 7- amino-actinomycin D (Sigma-Aldrich) and analyzed by flow cytometry.

### Quantitative real-time PCR using TaqMan chemistry

CTL were incubated with uninfected or HSV-infected fibroblasts for 4 h, removed by aspiration, and incubated for 5 h at 37°C. In the acyclovir group, fibroblasts were treated with 50 μM acyclovir for 2 h before infecting with HSV for 6 h. Removed CTL were then incubated with CD8-conjugated Dynabeads (Dynal A.S., Oslo, Norway) for 30-min rocking at 4°C and washed, as directed by the manufacturer. DNA was purified from uninfected fibroblasts, HSV-infected fibroblasts, and CD8-enriched CTL using DNeasy tissue kits (Qiagen, Valencia, CA). DNA was quantified by spectrophotometry and used as template in TaqMan real-time PCR with primers and fluorogenic probes specific for the HSV glycoprotein B gene or the cellular β-globin gene. Reactions were done on an ABI 7700 sequencer (Applied Biosystems, Foster City, CA), and the sequences
and concentrations of the primers and probes used were as previously described (41).

**Results**

**Effect of incubating CTL with fibroblasts infected with HSV-1 or HSV-2**

To evaluate the possibility that HSV-infected cells might send an inactivating signal to CTL, a confluent fibroblast monolayer was mock or HSV infected for 6 h and washed. Then added a CD8⁺ CTL clone that is restricted to MHC molecules not expressed on the fibroblasts (i.e., there was no killing of mock- or HSV-infected fibroblasts; data not shown). After incubating the HSV-infected fibroblasts and CTL for 4 h, ⁵¹Cr-labeled target cells were added, and the three-cell sandwich was incubated for an additional 5 h. At an MOI of 10 (i.e., 10 PFU of HSV per fibroblast) and a wide range of E:T ratios, both HSV-1-infected and HSV-2-infected fibroblasts, but not uninfected fibroblasts, markedly inhibited the ability of CTL to kill targets (Fig. 1A). We refer to this phenomenon as HSV-mediated inactivation, and it occurred at an MOI as low as 0.2 (Fig. 1B), suggesting a very robust negative signaling event between HSV-infected fibroblasts and CTL. To determine the kinetics of inactivation, the infection and CTL incubation times were varied independently. The ability of HSV-infected fibroblasts to cause inactivation occurred as soon as 2 h after infection and plateaued by 6 h with greater than 90% reduction of ⁵¹Cr release (Fig. 1C). At 6 h postinfection, inactivation was seen with as little as 1 h of CTL incubation with HSV-infected fibroblasts and was maximal by 3–4 h (Fig. 1D).

When CTL were incubated for 4 h with supernatant from fibroblasts that had been infected with HSV for 6 h, there was no effect on CTL cytotoxicity as measured by a standard ⁵¹Cr release assay (data not shown). Similarly, CTL were not inactivated when incubated with HSV-infected fibroblasts in a Transwell format that prevented contact between the two cell types (data not shown). These results suggest that CTL inactivation is not mediated by a soluble factor, but requires cell-to-cell contact.

To assess the durability of the inactivated phenotype, CTL were removed from HSV-infected fibroblasts by gentle aspiration. CTL removal also decreased the potential for CTL infection during subsequent steps and eliminated the possibility that HSV-infected fibroblasts might directly influence or infect target cells. A total of 80–90% of CTL was typically removed without removing fibroblasts, as determined by microscopic inspection and counting (data not shown). Removed CTL were incubated alone before adding targets, and they remained inactivated for at least 12 h (Fig. 2A). Treatment with IL-2 maintained the lytic ability of CTL added to mock-infected fibroblasts for up to 48 h, but CTL added to HSV-infected fibroblasts did not recover killing function even when treated with IL-2 (Fig. 2B).

In all subsequent experiments involving the HSV-mediated inactivation of CTL, fibroblasts were mock or HSV infected for 6 h at an MOI of 10, and CTL were incubated for 4 h with the mock- or HSV-infected fibroblasts. CTL from mock-infected fibroblasts (control) and HSV-infected fibroblasts (inactivated) were then removed from the fibroblasts before adding to target cells. When inactivated CTL were mixed with control CTL at ratios as high as 1:1, there was no suppression of lytic ability in the control CTL (data not shown).

Although chromium release is a traditional method for determining the cytotoxic capacity of CTL, more biologically relevant methods have recently become available that examine apoptotic

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**FIGURE 1.** HSV-mediated inactivation of CTL intensifies as HSV infection of fibroblasts increases and CTL incubation with HSV-infected fibroblasts increases. A. Fibroblasts were mock infected or infected with HSV-1 or HSV-2 for 6 h at an MOI of 10. The alloreactive CD8⁺ CTL clone SKH13 was then incubated for 4 h with HSV-infected fibroblasts. ⁵¹Cr-labeled LCL target cells were added to wells containing HSV-infected fibroblasts and CTL at variable E:T ratios. B. Fibroblasts were infected for 6 h with HSV-2 at variable MOIs, and CTL were incubated for 4 h with HSV-infected fibroblasts. C. Fibroblasts were infected with HSV-2 for variable times at an MOI of 10, and CTL were incubated for 2 h with HSV-infected fibroblasts. D. Fibroblasts were infected for 6 h with HSV-2 at an MOI of 10, and CTL were incubated with HSV-infected fibroblasts for variable times. E:T ratios of 2 were used for B–D. All data points represent triplicate values from three-cell sandwich assays measuring specific ⁵¹Cr release from LCL allogeneic target cells (HA-8 positive). ⁵¹Cr release from autologous targets (HA-8 negative) <1% (data not shown). Control CTL = CTL incubated on mock-infected fibroblasts. Inactivated CTL = CTL incubated on HSV-infected fibroblasts. A representative of two independent experiments is shown.

**FIGURE 2.** CTL remain inactivated when removed from HSV-infected fibroblasts and treated with IL-2. Fibroblasts were infected with HSV-2 (A) or HSV-1 (B) at an MOI of 10 for 6 h. The SKH13 (A) and the KSN (B) CTL clones were incubated for 4 h with HSV-infected fibroblasts and removed by gentle aspiration. Removed CTL were incubated for the times indicated without IL-2 (A) or in the presence or absence of IL-2 (B) at 20 U/ml before adding ⁵¹Cr LCL target cells at an E:T ratio of 3 for 6 h. The general decrease in all groups in CTL lysis over 48 h in culture is expected at the end of a 2-wk stimulation cycle. The percentage of inactivation in A represents the percentage of difference between specific chromium release in inactivated CTL and control CTL. A representative of two independent experiments is shown.
markers, such as activated caspase levels, in target cells (42). We used flow cytometry to assess activated caspase 3 levels in targets incubated with control or inactivated CTL. Target cells were first labeled with the integral membrane dye PKH26 to distinguish them from CTL. In apoptotic cells, activated caspase 3 levels increase. In agreement with the chromium release data, targets incubated with inactivated CTL had substantially less activated caspase 3 relative to those incubated with control CTL (Fig. 3A).

Caspase activation in target cells occurs primarily through the exocytosis of lytic granules from activated CTL. These granules contain perforin and granzymes A and B. The SKH13 CTL clone used this mechanism as well, because the addition of concanamycin, an agent that blocks granule exocytosis, prevented $^{51}$Cr release from target cells (data not shown). We evaluated granzyme A levels in inactivated CTL after incubation with targets. Consistent with our previous assessment of cytotoxicity, there was markedly more granzyme A remaining in the inactivated CTL relative to control CTL (Fig. 3B). Thus, HSV-inactivated CTL do not degranulate upon contact with target cells.

In addition to granule exocytosis, CTL control viral replication by synthesizing and secreting proinflammatory cytokines. To assess cytokine levels, CTL were treated with BFA, an agent that prevents secretion of proteins, during stimulation with target cells. Flow cytometric analysis revealed 90% less synthesis of IFN-γ and TNF-α in inactivated CTL relative to control CTL (Fig. 4). Therefore, CTL are inhibited in at least two mechanistically distinct effector pathways in our HSV-mediated inactivation model.

Cell surface markers involved in T cell costimulation, accessory signaling, and activation were measured on control and inactivated CTL. There was no detectable difference by flow cytometry for the following molecules: CD2, CD3, CD5, CD7, CD8, CD19, CD25, CD28, CD38, CD45, CD56, CD57, and HLA-DR (data not shown). An anti-CD94-blocking Ab added to CTL before incubating with HSV-infected fibroblasts did not alter inactivation, suggesting that the CD94-NKG2A molecules were also not involved. In addition, levels of the MIC, MICA and MICB, were similar on mock- and HSV-infected fibroblasts (data not shown).

**FIGURE 3.** HSV-inactivated CTL induce decreased apoptosis in target cells and retain more lytic granules after exposure to target cells. A, Targets were PKH26 labeled and added to CTL at an E:T ratio of 1 before staining for activated caspase 3. B, Unlabeled targets were added to CTL at an E:T ratio of 0.2. Cells were stained with Ab against CD8 and granzyme A and analyzed by flow cytometry gating for CD8-positive cells. A representative of three independent experiments is shown.

**FIGURE 4.** HSV-inactivated CTL synthesize decreased cytokine levels when stimulated with target cells. SKH13 CTL were inactivated, as previously described, with HSV-2-infected fibroblasts and removed before adding to target cells for 6 h at an E:T ratio of 0.2. BFA was added for the last 4 h of incubation between CTL and targets at a concentration of 10 μg/ml to prevent protein secretion. Cells were then stained with Ab against CD8, IFN-γ, and TNF-α and analyzed by flow cytometry. A representative of three independent experiments is shown.

**HSV-mediated inactivation does not require infection of CTL**

A possible explanation for inactivation of CTL in our model involves a negative signaling event between HSV-infected fibroblasts and uninfected CTL. This proposed mechanism is distinct from the inhibition of CTL function that occurs when CTL are infected with HSV. To determine whether viral replication was necessary in HSV-infected fibroblasts and to decrease the possibility of cell-to-cell spread of virus to CTL, fibroblasts were treated with acyclovir before infection. In HSV-infected cells, viral gene expression is divided temporally into three classes: immediate (α), early (β), and late (γ1 and γ2) genes. Acyclovir inhibits spread of virus from one infected cell to another by inhibiting viral DNA synthesis. As a result of inhibiting replication of viral DNA, γ2 gene expression is also inhibited. HSV-infected fibroblasts retained the capacity to inactivate CTL in the presence of 50 and 500 μM acyclovir (Fig. 5). As a follow-up to this finding, a carry-over

**FIGURE 5.** Acyclovir has a minimal effect on HSV-mediated inactivation of CTL. Fibroblasts were untreated or treated with 50 or 500 μM acyclovir for 2 h before a 6-h infection with HSV-2. CTL were added to HSV-infected or mock-infected fibroblasts for 4 h and then removed by aspiration. Control and inactivated CTL were added to target cells at an E:T ratio of 3 for 6 h. A representative of three independent experiments using both the SKH13 and KSN CTL clones is shown.
experiment was conducted. CTL were inactivated on acyclovir-treated, HSV-infected fibroblasts and removed. Inactivated CTL were then incubated with uninfected fibroblasts for 5 days. After 5 days, there were no cytopathological effects (i.e., microscopic changes in cell morphology that occur after infection with virus) on the fibroblasts incubated with inactivated CTL (data not shown). These results suggest that viral replication is not necessary for CTL inactivation by HSV-infected cells and that gB viral genes are not required for inactivation in our model.

Although acyclovir treatment substantially decreases the production of virus from infected cells, it does not rule out the possibility that HSV-inactivated CTL become infected in our model. The most sensitive technique available to detect the presence of HSV is real-time, quantitative PCR. Primers and probes against a viral gene (gB) and a cellular gene (β-globin) were used to determine the number of HSV copies per cell in HSV-infected fibroblasts and in inactivated CTL. Fibroblasts were untreated or treated with 50 μM acyclovir before infection. CTL were then inactivated as before, removed, and enriched using CD8-coupled magnetic beads to decrease possible contamination with HSV-infected fibroblasts. Although fibroblasts treated with acyclovir and infected with HSV were capable of inactivating CTL, we detected only 1 copy of HSV per CTL in this group, a value that approached the mock-infected background level of 1 copy of HSV per 29 fibroblasts (Table I). In the group without acyclovir treatment, HSV-infected fibroblasts contained 4344 copies of HSV per cell. CTL incubated on HSV-infected fibroblasts contained 6.5 copies of HSV per cell (Table I), suggesting that cell-to-cell spread of HSV from infected fibroblasts to CTL was a relatively rare event even in the absence of acyclovir. In summary, it is clear that HSV-mediated inactivation of CTL can occur in the absence of CTL infection in our model.

**Analysis of apoptosis in HSV-inactivated CTL**

The same numbers of CTL were recovered by aspiration from HSV-infected fibroblasts as from uninfected fibroblasts, and trypan blue staining revealed no evidence of preferential loss of viability in CTL after contact with HSV-infected fibroblasts (data not shown). However, this is a crude indicator of cell health. Therefore, activated caspase 3 levels in CTL were measured by flow cytometry to determine whether apoptosis played a role in our inactivation model. The percentage of control and inactivated CTL staining positive for activated caspase 3 was virtually equal (Fig. 6A). Another indicator of programmed cell death is the loss of mitochondrial membrane potential. CMXRos is a charge-sensitive fluorochrome that binds to the inner mitochondrial membrane and decreases in fluorescence as membrane potential is lost. In our model, HSV-inactivated CTL had a mitochondrial potential very similar to control CTL, whereas CTL treated with staurosporine, an agent that induces apoptosis through the mitochondrial pathway, showed a significant decrease in the CMXRos signal (Fig. 6B). In addition, flow cytometry revealed no detectable difference in CD95 (Fas) levels or in binding of 7-amino actinomycin D to inactivated and control CTL (data not shown). Taken together, these results suggest no evidence of increased programmed cell death in CTL inactivated by HSV-infected cells.

**Stimulating HSV-inactivated CTL through the TCR or with a phorbol ester plus ionomycin**

Our results demonstrated that HSV-inactivated CTL were not apoptotic nor were they infected with HSV. Furthermore, the inactivated phenotype was sustainable and not altered by IL-2 treatment (Fig. 2). Incubation of inactivated CTL with SEB, a superantigen that functions through TCR complex triggering, led to an increase in IFN-γ synthesis in control, but not inactivated CTL (Fig. 7A). Similarly, control, but not inactivated CTL were capable of lytic granule release via redirected lysis using anti-CD3 Ab-labeled CTL and 51Cr-labeled P815 cells that express abundant FcyR (Fig. 7B). In contrast, treatment of inactivated CTL with PMA plus ionomycin, a non-TCR method of stimulation, resulted in the production of IFN-γ levels comparable with control CTL (Fig. 7C). Taken together, these findings suggest that although HSV-inactivated CTL cannot be stimulated through the TCR, they

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**Table I. Quantitation of HSV levels in fibroblasts and CTL using real-time PCR**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>No. HSV Copies/Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock-infected fibroblasts</td>
<td>0.04</td>
</tr>
<tr>
<td>CTL incubated on HSV-FB + acyclovir</td>
<td>0.16</td>
</tr>
<tr>
<td>HSV-infected fibroblasts (HSV-FB)</td>
<td>4334</td>
</tr>
<tr>
<td>CTL incubated on HSV-FB</td>
<td>6.5</td>
</tr>
</tbody>
</table>

*TaqMan chemistry used previously published primers and probes against HSV glycoprotein B (gB) gene and the cellular β-globin gene (41).*

* Fibroblasts, untreated or treated with 50 μM acyclovir, were infected for 6 h with HSV-2 or mock-infected. CTL were added for 4 h and removed, then incubated for 5 h alone. CTL were enriched using CD8-coupled magnetic beads.

* DNA was extracted from cells and used as template. Number of HSV copies was determined by C\textsubscript{i} values for gB at a given template dilution, and number of cells was determined by C\textsubscript{j} values for β-globin at the same template dilution.*
are viable and retain the capacity to functionally respond to other signaling pathways.

Effect of UV irradiation and CHX on HSV-mediated inactivation

To determine whether the input virion was sufficient to inactivate CTL, HSV was UV irradiated before infecting fibroblasts. At 0.1 J of UV irradiation, virus was still capable of cell entry (data not shown), but inactivation of CTL was virtually abolished (Fig. 8A). This indicates that de novo viral protein synthesis is required to inactivate CTL. To confirm these findings, CHX was used to independently inhibit translation in fibroblasts, CTL, and target cells. Inactivation of CTL by HSV-infected fibroblasts was done, as previously described, except that each cell type was first treated with 10 µg/ml CHX. Although there was no effect on HSV-mediated inactivation when CTL or targets were exposed to CHX, treating the fibroblasts with CHX before infection abolished HSV-mediated inactivation (Fig. 8B). Therefore, protein synthesis is necessary in HSV-infected fibroblasts, but not in CTL in order for inactivation to occur.

Analysis of HSV deletion-rescue pairs in HSV-mediated inactivation of CTL

To identify viral genes that HSV-infected cells require to mediate inactivation, we used deletion-rescue pairs derived from the HSV-1 F parent strain. Fibroblasts infected with viruses containing deletions in U4 or UL54 were incapable of inactivating CTL, while deletion of US12 did not alter HSV-mediated inactivation (Fig. 9). Deletion of US5 did not prevent inactivation. However, deletion of US3 significantly decreased the ability of HSV-infected fibroblasts to inactivate CTL (Fig. 9, p < 0.005 using paired Student’s two-tailed t test).

Discussion

Our results demonstrate that HSV-infected cells engage a powerful signaling pathway in CD8+ CTL and dramatically inhibit their
effector functions. CTL inactivated by HSV-infected cells lose their ability to release cytolytic granules and synthesize cytokines when triggered through the TCR. The inactivated phenotype is durable and generalizable as it occurs in every CD8+ and CD4+ CTL clone tested in our lab regardless of Ag specificity (G. Zahariadis and K. R. Jerome, unpublished data). Our findings suggest that a ligand expressed on HSV-infected cells mediates the inactivation signal by binding an inhibitory receptor expressed on CTL. Two pieces of evidence support this. First, several techniques suggest that CTL can be inactivated without being infected. Second, the inactivation signal in our model requires cell-to-cell contact, as demonstrated by the loss of inactivation in supernatant transfer and Transwell experiments. This point is critical, as soluble factors, such as IFN-γ, can be secreted by virus-infected cells leading to altered CTL function (42, 43). In addition, our findings indicate that the inactivating receptor is expressed constitutively on CTL, because inhibition of new protein synthesis in CTL does not prevent inactivation. Finally, the inactivating receptor inhibits effector function without affecting cell viability, as inactivated CTL are not apoptotic and can respond to PMA. PMA is thought to function by activating the protein kinase C pathway (44), which suggests that the inactivating signal inhibits TCR signaling proximal to protein kinase C.

To rule out known mechanisms of CTL inhibition as an explanation for inactivation in our model, we examined several possibilities. Whereas IL-2 treatment restores CTL function in traditional anergy models (45), IL-2 does not restore function in our inactivation model. The inactivated phenotype is not transferable to protein kinase C.

The use of deletion-rescue pairs revealed some of the viral genes required for HSV-infected fibroblasts to inactivate CTL. The α trans-activating genes α4 and U54 are required for the expression of numerous β and γ genes. Not surprisingly, deletion of either gene abolished HSV-mediated inactivation, while deletion of U512, a non-trans-activating α gene, had no effect. From these results, it was clear that HSV-infected fibroblasts required one or more downstream viral genes (β or γ) to transmit the inactivating signal to CTL. Because our lab has been studying the roles that U53 and U55 play in protecting virally infected cells from undergoing apoptosis, HSV deletions for these genes were available and tested. As predicted from experiments using acyclovir in which γ gene expression should have been minimal, but inactivation still occurred, deletion of the γ gene U55 did not affect inactivation. However, deletion of the β gene U53 diminished the inactivation signal to a level comparable to the α4 or U54 deletions.

There are several known mechanisms by which U53 might be influencing the ability of HSV-infected cells to inactivate CTL. U53 reportedly phosphorylates both viral and cellular proteins, affecting their expression and function. Known viral substrates for U53 include the immediate early regulatory proteins ICP22 and U51.5, as well as U534, and U535 (56–58). U53 has also been shown to inhibit apoptosis in HSV-infected cells by modifying cellular proteins (22, 59). For example, U53 can posttranslationally modify Bad, a proapoptotic member of the Bcl-2 family, and prevent it from activating programmed cell death (60).

The significance of U53 in our model of CTL inactivation is illustrated by an in vivo model of murine HSV-2 genital herpes (61). In this model, there was more rapid viral clearance in mice infected with a U53-deficient mutant relative to a U52 mutant or wild-type virus. In addition, the U53-deficient mutant produced an immune response with increased number of CD8+ T cells and increased levels of secreted IFN-γ. This finding was largely attributed to the known antiapoptotic function of U53. However, our findings shed new light on this model. It is possible that the enhanced CTL response in the U53-deficient mutant was secondary to a loss of inactivation of CTL, suggesting that the inactivating signal from HSV-infected cells might be an important feature in the successful ability of HSV to infect, replicate, and persist in vivo.

We report that U53 expression in HSV-infected cells is necessary to transmit the inactivating signal. We have not established that U53 expression is sufficient to inactivate CTL. The U53 amino acid sequence does not have a predicted transmembrane domain or a signal sequence or GPI linkage. Therefore, U53 is unlikely to be found on the cell surface and probably does not act directly as the inactivating ligand. A more likely role for U53 is to modify, possibly phosphorylate, a viral or cellular protein functioning as the inactivating ligand. Identifying the ligand on U53-expressing, HSV-infected cells responsible for inactivating CTL could provide a means of specifically manipulating CTL function. This, in turn, could lead to the ability to stimulate dysfunctional CTL that exist in persistent viral infections and tumor states, or alternatively to promote inactivation in graft-vs-host disease, transplant rejection, and autoimmune states.
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References


