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Apoptosis and antigen receptor function in T and B cells following exposure to herpes simplex virus

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Abstract

T cells are an essential component of the immune response against herpes simplex virus (HSV) infection. We previously reported that incubation of T cells with HSV-infected fibroblasts inhibits subsequent T cell antigen receptor signal transduction. In the current study, we found that incubation of T cells with HSV-infected fibroblasts also leads to apoptosis in exposed T cells. Apoptosis was observed in Jurkat cells, a T cell leukemia line, and also in $CD4^+$ cells isolated from human peripheral blood mononuclear cells. Direct infection of these cells with HSV also resulted in apoptosis. Clinical isolates of both HSV type 1 and 2 induced apoptosis in infected T cells at comparable levels to cells infected with laboratory strains of HSV, suggesting an immune evasion mechanism that may be clinically relevant. Further understanding of these viral immune evasion mechanisms could be exploited for better management of HSV infection.

Keywords: Herpes simplex virus; Apoptosis; T cells; B cells; T cell antigen receptor

Introduction

Effective cell-mediated immunity is critical in control of herpes simplex virus (HSV) infection (Rinaldo and Torpey, 1993; Schmid and Rouse, 1992), and severe HSV disease can occur in immunocompromised patients (Herget et al., 2005; Kusne et al., 1991). HSV is characterized by a life-long infection with intermittent reactivations in immunocompetent people, and reactivation can occur on more than 75% of days in some individuals (Wald et al., 1997). The pathology and epidemiology of HSV reflect the ability of the virus to circumvent the host's immune system. Although the immune system in immunocompetent individuals will ultimately control the infection, the ability of HSV to evade the local immune responses for a limited time allows the virus to establish latent infection and reactivate for the life of the human host.

HSV uses multiple strategies to protect infected epithelial cells from the host's immune defenses. For example, HSV inhibits the major histocompatibility complex class I and II antigen presentation pathways (Favoreel et al., 2000; Koelle and Corey, 2003). Inhibition of these pathways protects infected cells from recognition by $CD8^+$ and $CD4^+$ T cells respectively. Other examples of immune evasion mechanisms include several HSV factors that have been reported to inhibit apoptosis and maintain the viability of infected cells (Aubert and Blaho, 2001; Aubert and Jerome, 2003; Goodkin et al., 2004). Identified factors include U_S3 protein kinase (Jerome et al., 1999; Leopardi and Roizman, 1996; Leopardi et al., 1997; Munger et al., 2001; Murata et al., 2002; Purves et al., 1987), glycoprotein J (Jerome

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et al., 2001a; 1999; Zhou et al., 2000), glycoprotein D (Zhou et al., 2003, 2000; Zhou and Roizman, 2001; Zhou and Roizman, 2002a, 2002b), ICP10 protein kinase (Perkins et al., 2003, 2002a, 2002b), and latency-associated transcripts (Ahmed et al., 2002; Gupta et al., 2006; Inman et al., 2001; Perng et al., 2000). Efficiency of inhibition depends on the specific viral factor and the apoptotic stimulus. Our laboratory previously reported that deletion of the U_S3 gene markedly reduced inhibition of ultraviolet light induced apoptosis in HSV-infected cells (Jerome et al., 1999), while expression of the U_S5 gene (glycoprotein J) protected cells from granzyme B and Fas-mediated cell death (Jerome et al., 2001a). Such findings probably reflect the apoptotic pathway targeted by each anti-apoptotic viral factor.

Despite numerous studies characterizing the anti-apoptotic mechanisms of HSV (Aubert and Blaho, 2001; Aubert and Jerome, 2003; Goodkin et al., 2004), immune cells infected with HSV undergo apoptosis. HSV infection of T cells can directly lead to apoptosis (Ito et al., 1997a, 1997b; Pongpanich et al., 2004). Infection of HSV-specific cytotoxic T lymphocytes (CTL) has also been reported to result in more rapid apoptosis via "fratricide" (killing of each other) because major histocompatibility complex I antigen presentation is not blocked in CTL following HSV infection (Raftery et al., 1999). In addition, monocytoid cells (Mastino et al., 1997), dendritic cells (Bosnjak et al., 2005; Jones et al., 2003), and macrophages (Fleck et al., 1999) have been reported to show signs of apoptosis following HSV infection. These findings indicate that induction of apoptosis in key immune effector cells may be an important mechanism of viral immune modulation.

In vivo, T cells are mostly likely to encounter HSV via infected keratinocytes during trafficking of HSV-specific T cells to sites of virus reactivation (Posavad et al., 1998). This laboratory and others have previously demonstrated modulation of T cell function when T cells are exposed to HSV-infected fibroblasts *in vitro* (Posavad et al., 1993; Posavad and Rosenthal, 1992; Sloan et al., 2003, 2006). Specifically, cytolytic activity (Posavad et al., 1993; Posavad and Rosenthal, 1992; Sloan et al., 2003) and T cell antigen receptor (TCR) signal transduction (Sloan et al., 2003, 2006) are impaired in T cells following incubation with HSV-infected fibroblasts. Our study suggested that increased apoptosis was not present in CTL within the first 5 h following an exposure to HSV-infected fibroblasts (Sloan et al., 2003). In addition, while we previously reported that clinical HSV strains did not lead to caspase-3 or -8 activation in infected Jurkat cells (Jerome et al., 2001b), a recent report demonstrated apoptosis induction in Jurkat cells at later time points after infection with laboratory strains of HSV (Pongpanich et al., 2004).

To determine if induction of apoptosis is a potential mechanism by which HSV modulates T cell function, we have carried out more detailed studies to detect apoptosis in Jurkat cells and $CD4^+$ T cells isolated from human peripheral blood mononuclear cells (PBMC) that are either exposed to HSV-infected fibroblasts or directly infected with laboratory strains of HSV. We investigated whether induction of apoptosis is an attribute of laboratory HSV strains by comparing induction of apoptosis in Jurkat cells infected with laboratory strains against cells infected with clinical isolates of HSV. We also investigated whether apoptosis could be seen in B cells by studying Ramos cells, a B cell lymphoma line. Finally, two potential HSV immune modulation mechanisms, induction of apoptosis and inhibition of TCR signaling, were studied simultaneously to discern the relationship between the two processes.

Results

Jurkat cells directly infected with HSV undergo apoptosis

To investigate whether HSV induces apoptosis in T cells, we first evaluated apoptotic markers in Jurkat cells directly infected with HSV-2. Jurkat cells were infected with HSV-2 strain HG52 at a multiplicity of infection (MOI) of 5 and analyzed for apoptosis and necrosis at 6 h and 24 h post infection (p.i). For this purpose, we used annexin V, a protein that binds with high affinity to phosphatidylserine residues that become exposed on the surface of apoptotic cells. Cells show reactivity with annexin V before the plasma membrane loses its ability to exclude a dye such as propidium iodide (PI). Thus, by staining cells with a combination of fluorescently labeled annexin V and PI, it is possible to distinguish viable cells (annexin V^-PI^-), early apoptotic cells (annexin V^+PI^-), and late apoptotic or necrotic cells (annexin V^+PI^+). Our results indicated that HSV-2 infection induced apoptosis in Jurkat cells that could be observed at 6 h p.i. (Fig. 1A). The percentage of early apoptotic cells was increased at 24 h p.i., with the average of 41% of HSV-infected cells that were annexin V^+PI^- compared with 8% of mock-infected cells. There was also a corresponding increase in annexin V^+PI^+ cells in HSV-infected cells at 24 h, most likely representing an increase in late apoptotic cells.

We sought further evidence of apoptosis in HSV-2-infected Jurkat cells by determining the percentage of cells with activated caspase-3. Cells were labeled with antibody against activated caspase-3 and analyzed by flow cytometry. Compared to a baseline of 9% in mock-infected cells, 19% of HSV-infected cells demonstrated activated caspase-3 at 6 h p.i., and the percentage was increased to 41% at 24 h p.i. (Fig. 1B). Thus, HSV-2 infection of Jurkat cells also induced caspase-3 activation.

To determine dose and time dependence of apoptosis induction, Jurkat cells were infected with HSV-2 strain HG52 at different MOI and analyzed for annexin V binding at 6, 12, 18, and 24 h p.i. A step-wise increase in apoptotic cells was noted over time in infected cells (Fig. 1C). A minimal increase in the percentage of annexin V^+ cells was observed above MOI of 5, suggesting that the viral infection of Jurkat cells leads to apoptosis rather than non-viral stimulation from the inoculum.

$CD4^+$ cells isolated from human PBMC undergo apoptosis with HSV infection

To assess if our findings with Jurkat cells are applicable to primary T cells, $CD4^+$ cells were isolated from human PBMC following stimulation with phytohemagglutinin and IL-2.

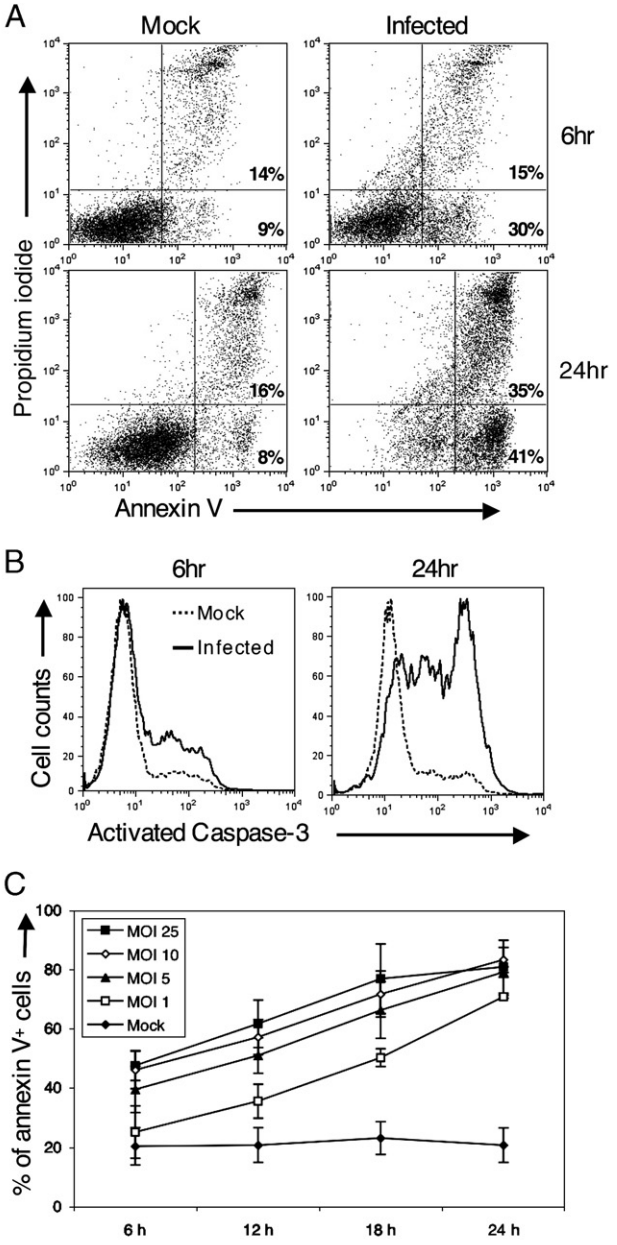

Fig. 1. Induction of apoptosis by HSV-2 infection in Jurkat cells. (A) Jurkat cells were mock infected or infected with HSV-2 HG52 at an MOI of 5 and analyzed for apoptosis with annexin V and PI binding at 6 h and 24 h p.i. One representative flow cytometry experiment out of 4 independent experiments is shown. Mean percentages of annexin V^+PI^- (early apoptotic) and annexin V^+PI^+ (late apoptotic or necrotic) cells of the 4 independent experiments are noted. (B) Flow cytometry analysis of mock-infected (dashed line) and HSV-2-infected (solid line) cells stained with anti-activated caspase-3 antibody at 6 h and 24 h p.i. (C) Percentages of annexin V^+ Jurkat cells over time after infection with varying MOI of HSV-2. Jurkat cells were mock infected or infected with HSV-2 at MOI of 1, 5, 10, and 25. Percentages of annexin V^+ Jurkat cells were analyzed by flow cytometry at 6, 12, 18, and 24 h p.i. Bars represent the standard error of two independent experiments.

Isolated $CD4^+$ cells were infected with HSV-2 strain HG52 at an MOI of 5 and analyzed for annexin V and PI binding at 24, 48, and 72 h p.i. The percentage of annexin V^+ cells (annexin V^+PI^- and annexin V^+PI^+) was 32% in HSV-infected $CD4^+$ cells compared with 28% in mock-infected cells at 24 h p.i (Fig. 2A). At 48 h, 49% of HSV-infected $CD4^+$ cells were annexin V^+ compared to 32% in mock-infected cells, while 52% of HSV-infected cells and 40% of mock-infected cells were annexin V^+ at 72 h. The majority of the annexin V^+ population in mock-infected cells was also PI^+, and the percentage of annexin V^+PI^+ cells increased in both the mock and HSV-2-infected cells over the time, likely reflecting the spontaneous death that occurs with primary $CD4^+$ cells in culture. This background cell death may explain the lack of statistical differences between mock- and HSV-infected cells when comparing the percentages of annexin V^+ cells. In contrast, marked increases in the percentages of early apoptotic cells (annexin V^+PI^-) were observed in HSV-infected cells compared to mock-infected cells at all time points (Fig. 2B). Thus, the

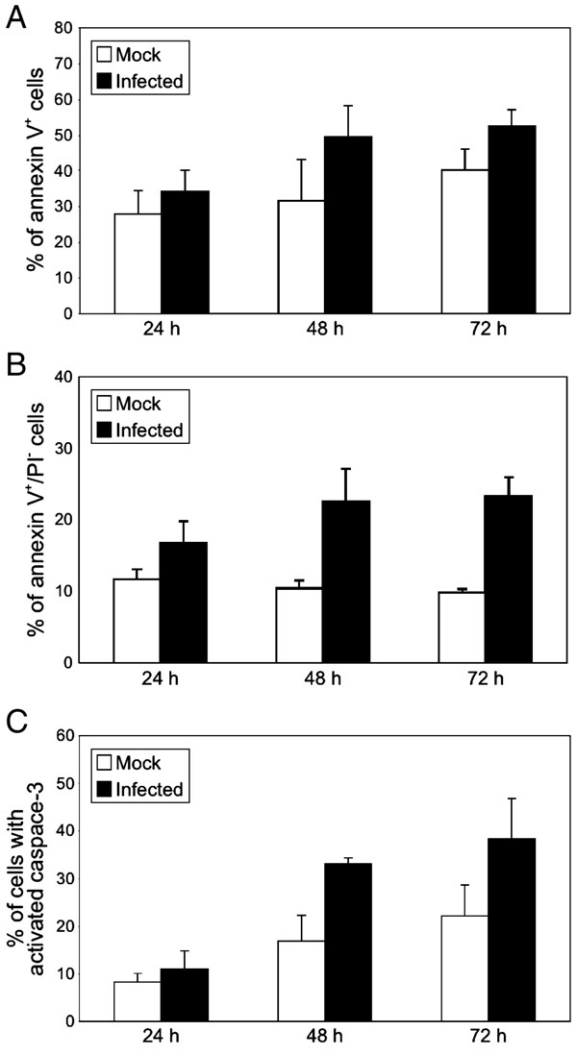

Fig. 2. $CD4^+$ cells infected with HSV-2 undergo apoptosis. $CD4^+$ cells isolated from human PBMC were mock infected or infected with HSV-2 HG52 at an MOI of 5 and analyzed for apoptosis at 24 h, 48 h, and 72 h p.i. (A) Percentages of annexin V^+ cells by flow cytometry with standard error bars of 3–4 independent experiments. (B) Percentages of annexin V^+/PI^- cells by flow cytometry analysis with standard error bars of 3–4 independent experiments. (C) Percentages of cells staining with anti-activated caspase-3 antibody at 24 h, 48 h, and 72 h p.i. with standard error bars of 3 independent experiments.

increase in the annexin V^+PI^- population accounted for the majority of increase in the percentage of annexin V^+ cells in HSV-infected cells compared to mock-infected cells.

To verify our findings, we analyzed mock- and HSV-infected $CD4^+$ cells for the presence of activated caspase-3. Cells were labeled with antibody against activated caspase-3 and analyzed by flow cytometry at 24, 48, and 72 h p.i. Similar to the results using annexin V, a slight increase in cells with activated caspase-3 was observed at 24 h p.i. for HSV-infected cells compared to mock-infected cells, with larger increases detected at 48 and 72 h p.i. (Fig. 2C).

Primary isolates of HSV-1 and HSV-2 induce apoptosis in Jurkat cells

Primary clinical isolates of HSV-1 and HSV-2 were evaluated for their apoptotic activity in infected Jurkat cells to rule out the possibility that adaptation to laboratory growth was responsible for the apoptotic activity. Jurkat cells were infected with HSV at an MOI of 5 with clinical isolates of HSV-1 and HSV-2 obtained from the University of Washington diagnostic virology laboratory. Laboratory strains HSV-1 F and HSV-2 HG52 were utilized as controls. At 24 h p.i., all clinical isolates of HSV showed induction of apoptosis at comparable levels to laboratory strains, as measured by annexin V binding (Fig. 3).

Jurkat cells exposed to HSV-infected fibroblasts undergo apoptosis

In vivo, T cells are most likely to encounter HSV via infected keratinocytes (Posavad et al., 1998). A previous report from this laboratory suggested that increased apoptosis was not present in CTL within the first 5 h following an exposure to HSV-infected fibroblasts despite demonstrated inhibition of cytolytic activity and TCR signaling block (Sloan et al., 2003). Given our findings in HSV-infected Jurkat cells, we addressed whether Jurkat cells that are exposed to HSV-infected fibroblasts undergo apoptosis at time points later than the first 5 h. Since we previously demonstrated that acyclovir, a viral DNA synthesis inhibitor, had no effect on HSV-mediated inhibition of CTL cytolytic activity (Sloan et al., 2003), we conducted the entire study in the presence of acyclovir to minimize infection of Jurkat cells by replication competent virus.

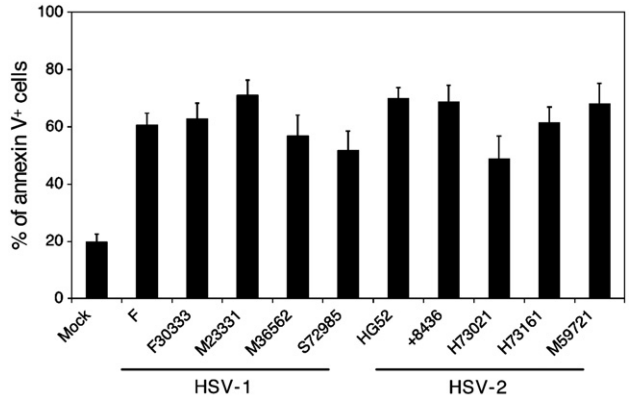

Fig. 3. Induction of apoptosis after infection by laboratory or clinical isolates of HSV. Annexin V binding was analyzed by flow cytometry in Jurkat cells that were mock infected or infected with HSV for 24 h. Shown are mock cells and cells infected with HSV-1 laboratory strain F, clinical isolates of HSV-1, HSV-2 laboratory strain HG52, and clinical isolates of HSV-2. Bars represent the standard error of 3 independent experiments.

The series of experiments was conducted in the same time frame as our previous experimental model (Sloan et al., 2003). Human primary fibroblasts were either mock-infected or infected with HSV-2 HG52 for 6 h. Jurkat cells were exposed to the fibroblasts for 4 h. Jurkat cells were then harvested and analyzed for apoptosis at 0, 6, 12, and 24 h post exposure. We observed a minimal difference in the percentage of apoptosis in Jurkat cells exposed to HSV-infected fibroblasts at 0 h (i.e., after 4-h incubation with infected fibroblasts) by annexin V and PI staining as compared with the control cells (Fig. 4A). A higher percentage of annexin V^+ cells was clearly appreciated at 6 h for Jurkat cells exposed to HSV-infected fibroblasts, and we observed a gradual increase in the percentage of apoptotic cells over time to a maximum of 70% at 24 h (Fig. 4A). Consistent with our finding in CTL (Sloan et al., 2003), a minimal difference in the percentage of cells with activated caspase-3 was noted at 0 h (Fig. 4B). Similar to the annexin V and PI staining, a higher percentage of cells with activated caspase-3 was noted at 6 h in the cells exposed to HSV-infected fibroblasts, and caspase-3 activation increased over 24 h (Fig. 4B).

To confirm the activity of caspase-3, we investigated the cleavage of the DNA repair enzyme poly(ADP-ribose) polymerase (PARP) from a 115-kDa form to an 85-kDa fragment. As shown in Fig. 4C, PARP is mostly expressed as a single 115-kDa polypeptide in Jurkat cells exposed to mock-infected fibroblasts. Some amount of baseline cleavage products could be appreciated in Jurkat cells exposed to mock-infected fibroblasts, consistent with a baseline apoptosis seen with annexin V and activated caspase-3 staining. In Jurkat cells exposed to HSV-infected fibroblasts, cleavage of PARP is demonstrated by appearance of the 85-kDa fragment starting at 6 h post harvest with corresponding decreases in the 115-kDa fragment. The processing of this protein was incomplete at all examined time points, and full-length protein was still detected at 24 h.

Inhibition of TCR signaling in Jurkat cells following exposure to HSV-infected fibroblasts as previously described in our model (Sloan et al., 2006) was confirmed by measuring the percentage of cells with calcium mobilization above baseline following stimulation of TCR with anti-CD3 monoclonal antibody (OKT-3). A 70% decrease in a percentage of cells able to flux calcium was observed following TCR trigger in HSV-exposed Jurkat cells at 0 h (Fig. 4D), and the inhibition remained unchanged at 0, 6, 12, and 24 h (data not shown).

$CD4^+$ cells isolated from human PBMC undergo apoptosis following exposure to HSV-infected fibroblasts

To corroborate our findings with Jurkat cells, human $CD4^+$ cells from PBMC were exposed to HSV-infected fibroblasts at

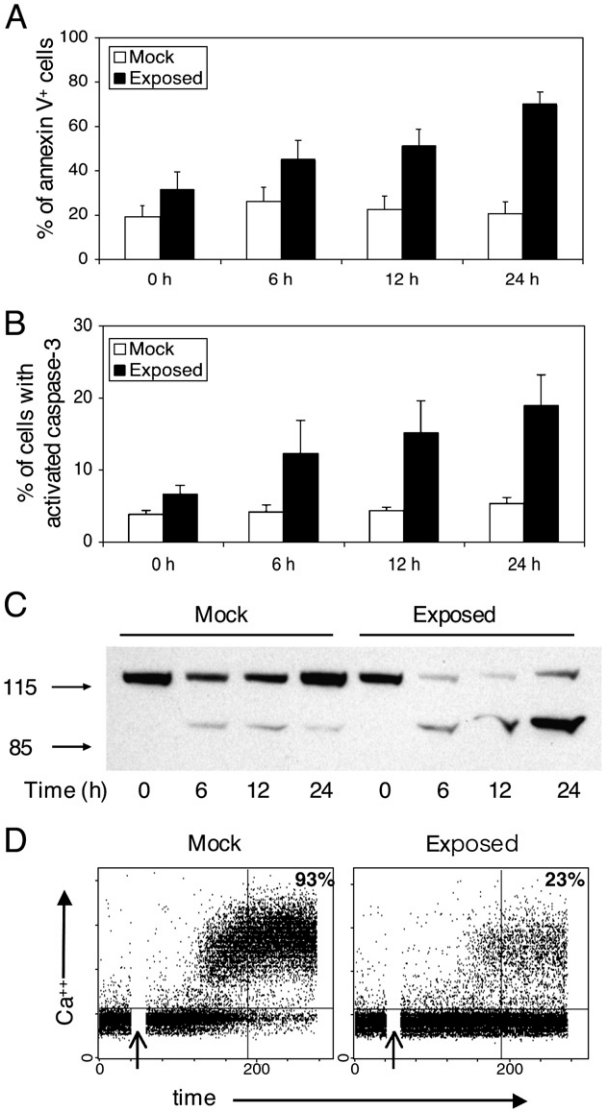

Fig. 4. Jurkat cells exposed to HSV-2-infected fibroblasts undergo apoptosis and show inhibition of TCR signaling. Percentages of Jurkat cells that are annexin V^+ (A) and activated caspase-3 positive (B) by flow cytometry at 0, 6, 12, and 24 h post exposure to mock- or HSV-2-infected fibroblasts. Bars represent standard error of 3–6 independent experiments. (C) Full-length PARP (115 kDa) and PARP cleavage products (85 kDa) in Jurkat cells exposed to mock and HSV-2-infected fibroblasts at 0, 6, 12, and 24 h post exposure. (D) Calcium mobilization following TCR stimulation of Jurkat cells exposed to mock- or HSV-2-infected fibroblasts at 0 h post exposure. Calcium mobilization was measured by 400-nm to 510-nm fluorescence emission ratio by flow cytometry. Numbers in the upper right quadrant of each plot indicate the percentage of Jurkat cells able to flux calcium as defined by greater than 95 percentile of unstimulated baseline prior to addition of OKT-3 (vertical arrows). One representative experiment out of 3 independent experiments is shown.

the same time intervals as above and analyzed for annexin V and PI binding at 24 h post harvest. 33% of $CD4^+$ cells were annexin V^+PI^- at 24 h following exposure to HSV-infected fibroblasts as compared to 4% of cells exposed to mock-infected fibroblasts (Fig. 5). Background necrosis and/or apoptosis in $CD4^+$ cells exposed to mock-infected fibroblasts were markedly lower compared to when $CD4^+$ cells were mock infected in the absence of fibroblasts (Fig. 2A), allowing a clearer demonstration of the difference between the mock-exposed and HSV-exposed cells in this set of experiments.

Inhibition of TCR signaling is observed in both apoptotic and non-apoptotic HSV-2-infected Jurkat cells

To delineate the relationship between induction of apoptosis and TCR signaling block, we evaluated the inhibition of TCR signaling in apoptotic and non-apoptotic Jurkat cells following HSV-2 infection. Jurkat cells were infected with HSV-2 HG52 for 6 h prior to analysis. Cells were simultaneously stained with indo-1 and annexin V to evaluate calcium mobilization in apoptotic and non-apoptotic populations by flow cytometry. The apoptotic and non-apoptotic populations were separated by forward scatter and annexin V staining. Both apoptotic and non-apoptotic cells demonstrated inhibition of TCR signaling as measured by decreases in the percentage of cells mobilizing calcium following OKT-3 stimulation (Fig. 6).

Inhibition of apoptosis in Jurkat cells exposed to HSV-2-infected fibroblasts has no effect on TCR signaling block

To delineate further the interaction, if any, between induction of apoptosis and inhibition of TCR signaling, we evaluated the effect of a pan-caspase inhibitor, z-VAD-fmk, on Jurkat cells exposed to HSV-infected fibroblasts. In this series of experiments, Jurkat cells were incubated in medium containing 50 μM z-VAD-fmk or DMSO solvent control for 30 min before exposure to HSV-2-infected fibroblasts, throughout exposure,

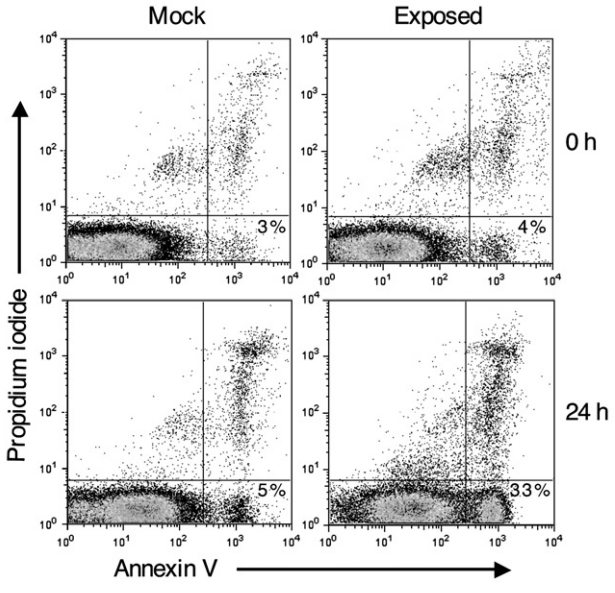

Fig. 5. Induction of apoptosis in $CD4^+$ cells exposed to mock- or HSV-infected fibroblasts. $CD4^+$ cells were exposed to mock or HSV-2-infected fibroblasts. $CD4^+$ cells were then collected and analyzed for apoptosis by annexin V and PI staining at 0 h and 24 h post exposure. Percentages of annexin V^+PI^- (early apoptotic) cells are shown for a representative flow cytometry analysis (out of 3 independent experiments).

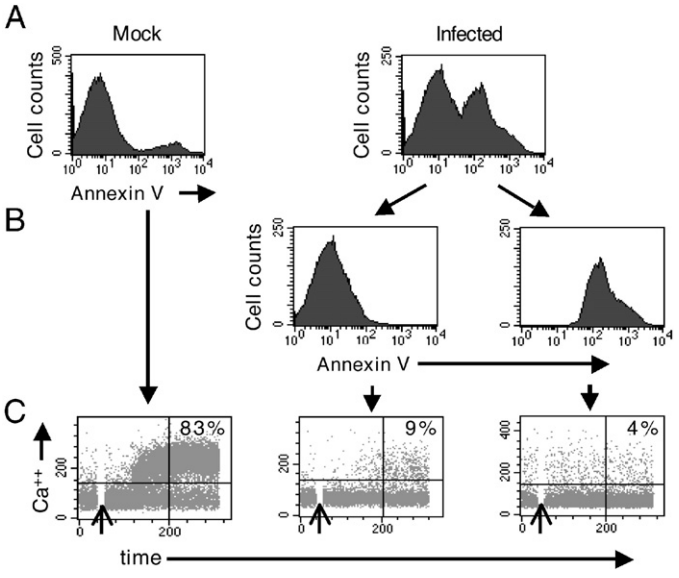

Fig. 6. Inhibition of TCR signaling pathway in apoptotic and non-apoptotic populations in HSV-2-infected Jurkat cells. Jurkat cells were infected with HSV-2 HG52 for 6 h prior to analysis. Cells were stained simultaneously with indo-1 and annexin V for detection of calcium flux in apoptotic and non-apoptotic populations. (A) Annexin V stain shows both apoptotic and non-apoptotic cells at 6 h p.i. (B) Apoptotic and non-apoptotic populations were gated individually and assessed for the ability to mobilize calcium by flow cytometric analysis. (C) Both populations demonstrate inhibition of TCR signaling pathway as shown by the small percentages of cells (upper right quadrants) able to mobilize calcium following OKT-3 stimulations (vertical arrows).

and post harvest. Cells were harvested at 0 h and 24 h post exposure and analyzed for apoptosis and calcium mobilization following TCR stimulation with OKT-3. Apoptosis in these cells was inhibited with z-VAD-fmk as measured by annexin V and PI staining at 24 h (Fig. 7A). However, z-VAD-fmk had no discernible effect on the TCR signaling block, which occurred to a similar degree in Jurkat cells with or without the caspase inhibitor (Fig. 7B).

To verify this finding, we also investigated phosphorylation status of extracellular signal-regulated protein kinases (ERK) after stimulation with OKT-3 in these cells. ERK is one of the intermediary molecules that become tyrosine phosphorylated following activation of TCR signaling cascade. We previously demonstrated that phosphorylation of ERK following OKT-3 stimulation is inhibited in T lymphocytes exposed to HSV-infected fibroblasts (Sloan et al., 2006). As shown in Fig. 7C, phosphorylation of ERK is inhibited in Jurkat cells exposed to HSV-infected fibroblasts despite the presence of z-VAD-fmk.

Ramos cells exposed to HSV-2-infected fibroblasts undergo apoptosis without inhibition of BCR signaling

We explored whether our findings in T cells were more broadly applicable to all lymphocytes by investigating if apoptosis was present in B cells exposed to HSV-infected fibroblasts. The Epstein-Barr virus-negative human B cell lymphoma line Ramos was used in these experiments. By annexin V and PI staining, we observed an increased percentage of apoptotic cells at 24 h and 48 h in Ramos cells exposed to HSV-2-infected fibroblasts as compared to those cells exposed to mock-infected fibroblasts (Fig. 8A).

Previous studies have shown that stimulation of B cell antigen receptor (BCR) leads to apoptosis in Ramos cells with a peak at 48 h (An et al., 2003; Mackus et al., 2002). In agreement with these studies, BCR stimulation of Ramos cells exposed to mock-infected fibroblasts, using anti-human IgM antibody, resulted in apoptosis (data not shown). Similar results were seen with activated caspase-3 staining (data not shown). However, BCR stimulation of Ramos cells exposed to HSV-infected fibroblasts did not increase apoptosis beyond that of Ramos cells exposed to HSV-infected fibroblasts without BCR stimulation, suggesting that these pro-apoptotic stimuli are not additive (data not shown).

Previous studies from this and other laboratories have shown that inhibition of cytotoxic activity occurs in natural killer cells, lymphokine-activated killer cells, and CTL after exposure to HSV-infected fibroblasts (Confer et al., 1990; Posavad et al., 1993; Posavad and Rosenthal, 1992; Sloan et al., 2003; York

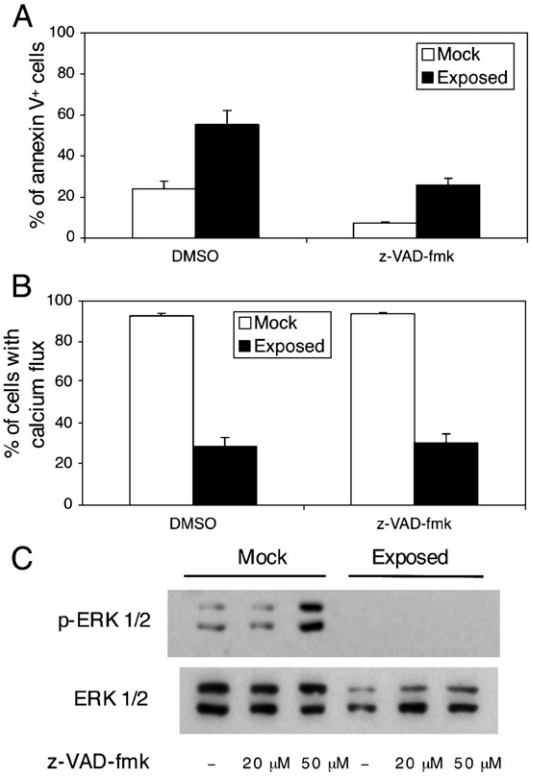

Fig. 7. z-VAD-fmk inhibits apoptosis without affecting inhibition of TCR signaling. (A) Percentages of annexin V^+ Jurkat cells with standard error bars of 3 independent experiments as measured by flow cytometry. The percentage of annexin V^+ cells decreases following exposure to HSV-2-infected fibroblasts in presence of 50 μM z-VAD-fmk compared to solvent control with DMSO. Bars represent the standard error of 3 independent experiments. (B) Inhibition of TCR signaling, as measured by decrease in the percentage of cells mobilizing calcium following TCR stimulation, does not change in the presence of z-VAD-fmk. Bars represent the standard error of 3 independent experiments. (C) Jurkat cells exposed to mock-infected fibroblasts demonstrated phosphorylation of ERK1/2 following TCR stimulation. Inhibition of phosphorylation in Jurkat cells exposed to HSV-2-infected cells was maintained in the presence of 20 μM or 50 μM z-VAD-fmk.

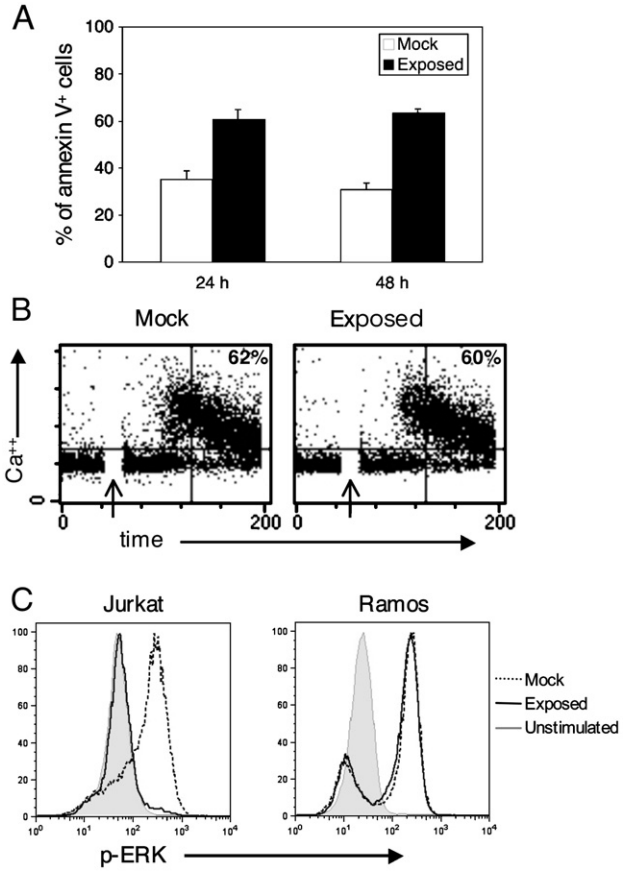

Fig. 8. Ramos cells undergo apoptosis without inhibition of BCR signaling pathway following exposure to HSV-2-infected fibroblasts. Ramos cells were exposed to mock or HSV-2-infected fibroblasts as described for Jurkat cells. (A) Percentages of annexin V^+ cells by flow cytometry analysis in Ramos cells exposed to mock or HSV-2-infected fibroblasts at 24 h and 48 h post exposure with standard error bars of 3 independent experiments. (B) Calcium mobilization following BCR stimulation of Ramos cells exposed to mock- or HSV-2-infected fibroblasts. Numbers in the upper right quadrant of each plot indicate the percentages of Ramos cells that were able to flux calcium. The percentages were determined by counting cells above the 95 percentile of unstimulated baseline prior to the addition of anti-human IgM antibody (vertical arrows). One representative experiment out of 7 independent experiments is shown. (C) Left panel: phosphorylation of ERK after TCR stimulation in Jurkat cells exposed to HSV-2-infected fibroblasts (solid line) compared to Jurkat cells exposed to mock-infected fibroblasts (dashed line). Jurkat cells without TCR stimulation (filled) are shown as control. Right panel: phosphorylation of ERK with BCR stimulation of Ramos cells following exposure to HSV-2-infected fibroblasts (solid line) compared to Ramos cells exposed to mock-infected fibroblasts (dashed line). Unstimulated Ramos cells (filled) are shown as control.

and Johnson, 1993). This laboratory subsequently demonstrated the inhibition of the TCR signaling pathway in T lymphocytes exposed to HSV-infected fibroblasts (Sloan et al., 2003, 2006). If a BCR signaling block were present in Ramos cells exposed to HSV-infected fibroblasts, such a block might explain why BCR stimulation did not result in increased apoptosis. Therefore, we assessed BCR signaling in Ramos cells following exposure to HSV-infected fibroblasts.

Two arms of BCR signaling cascade were examined: calcium mobilization and ERK phosphorylation. Ramos cells were exposed to mock-infected or HSV-infected fibroblasts. Calcium mobilization was determined following stimulation with anti-human IgM antibody. Ramos cells exposed to HSV-infected fibroblasts demonstrated virtually no change in calcium mobilization compared to control cells exposed to mock-infected fibroblasts (Fig. 8B). In contrast, similar experiments in Jurkat cells showed up to a 70% decrease in number of cells able to mobilize calcium following TCR stimulation with OKT-3 (Fig. 4D). Similarly, phosphorylation of ERK was not inhibited in Ramos cells following exposure to HSV-2-infected fibroblasts (Fig. 8C). Thus, although exposure to HSV-infected fibroblasts resulted in apoptosis of Ramos cells, inhibition of BCR signaling did not occur.

Discussion

In the present study, we showed that HSV infection results in apoptosis of Jurkat cells and $CD4^+$ cells isolated from human PBMC. Exposure to HSV-infected fibroblasts resulted in apoptosis of Jurkat cells, $CD4^+$ cells, and Ramos cells. In addition, we observed that clinical strains of HSV-1 and HSV-2 induced apoptosis in infected Jurkat cells at comparable levels to the laboratory strains. The findings of our current study suggest that an intriguing aspect of HSV modulation of host immune response is through induction of apoptosis in key immune effector cells. Given that most HSV-infected epithelial cells do not show classic signs of apoptosis because certain viral proteins act to inhibit the cell death process (Aubert and Blaho, 2001; Aubert and Jerome, 2003; Goodkin et al., 2004), the fact that HSV induces apoptosis in lymphocytes has implications for pathogenesis *in vivo*. Prevention of apoptosis in infected keratinocytes and simultaneous induction of apoptosis in local immune cells may tip the balance in favor of the virus, allowing its survival and propagation. Thus, the ability of HSV to kill T cells that are recruited to the site of reactivated lesions *in vivo* could play a part in maintaining the viability of these lesions and lengthening the time of viral shedding.

The precise mechanism(s) by which HSV induces apoptosis in exposed lymphocytes is not clear. Given that HSV-infected lymphocytes become apoptotic, we favor the idea that viral element(s) are responsible for induction of apoptosis. Thus, T cells that are exposed to infected fibroblasts may be induced to die by viral element(s) that are transferred during cell to cell contact. However, it is possible that lymphocytes could also be induced to die by exposure to cellular products from HSV-infected fibroblasts.

Previous studies indicated that apoptosis is initially induced following HSV infection of epithelial cells such as HEp-2 and is subsequently aborted by anti-apoptotic proteins (Aubert and Blaho, 2001; Aubert and Jerome, 2003; Goodkin et al., 2004). A previous report from our laboratory that suggested that at early time points after infection, clinical HSV strains protected Jurkat cells from apoptosis induced by anti-Fas antibody or UV radiation (Jerome et al., 2001b). However, in our current study we observed that clinical strains of HSV-1 and HSV-2 also induced apoptosis. Since the same HSV-1 clinical strains were used in both studies, we believe that this finding suggests that the HSV anti-apoptotic proteins are functional in T cells, but are

overwhelmed at later time points by the pro-apoptotic stimulus of viral infection. It may also be possible that an additional apoptosis triggering mechanism occurs in HSV-infected immune cells that bypass the viral anti-apoptotic mechanisms. Alternatively, certain viral pathways designed to inhibit apoptosis in other cell types may be inactive in lymphocytes, resulting in apoptosis.

Considering the mechanism of apoptosis induction by HSV in lymphocytes, it is important to note the differences in the extent and the temporal timing of apoptosis in different cell types in our study. In particular, primary $CD4^+$ cells isolated from human PBMC showed smaller increases in the percentages of apoptotic cells as compared to Jurkat and Ramos cells. It is possible that these $CD4^+$ cells are more resistant to apoptotic stimulus than the transformed cell lines. However, the percentage of apoptotic cells in Jurkat and $CD4^+$ cells were similar following an incubation with anti-Fas monoclonal antibody in our hands (data not shown), suggesting equal susceptibilities to stimulation of the Fas pathway in these two cell types. An alternative explanation may be that certain lineages of $CD4^+$ cells may be innately resistant to infection by HSV, and thus the pro-apoptotic viral element(s) may not be present in these cells. We are currently undertaking studies to decipher the precise mechanism of cell death in HSV-exposed T cells.

Given our findings of apoptosis in Jurkat cells exposed to HSV-2-infected fibroblasts, it was important to reexamine our findings of TCR signal block in similarly exposed Jurkat cells. CD95/Fas ligation, a common means of inducing apoptosis, also leads to inhibition of TCR signaling, as measured by calcium mobilization and ERK1/2 phosphorylation (Kovacs et al., 1999; Kovacs and Tsokos, 1995; Lepple-Wienhues et al., 1999; Yankee et al., 2001). However, CD95/Fas-mediated inhibition of TCR signal transduction is thought to be secondary to caspase-mediated cleavage of adaptor proteins (Yankee et al., 2001). Jurkat cells exposed to HSV-2-infected fibroblasts demonstrated inhibition of TCR signal transduction despite the presence of pan-caspase inhibitor, z-VAD-fmk, suggesting that inhibition is not mediated by caspases. Because the inhibition of apoptosis with z-VAD-fmk was not complete in Jurkat cells, we cannot completely rule out the possibility that small amounts of activated caspases could lead to inhibition of TCR signal transduction. In addition, earlier events in the apoptotic pathway may not be affected by z-VAD-fmk, and these events could mediate the inhibition of TCR signal transduction. However, TCR signal block in HSV-exposed Jurkat cells could be demonstrated before the cells showed signs of apoptosis, suggesting a temporal dissociation between the two outcomes. Thus, it seems likely that while caspase activation may contribute to the overall dysfunction of T cells, an additional mechanism leading to inhibition of the TCR signal must exist that is independent of caspase activation and subsequent apoptosis.

In conclusion, our results demonstrate that HSV induces apoptosis in both immortalized and primary lymphocytes. Apoptosis occurs in both infected T cells and T cells that are exposed to HSV-infected fibroblasts. Apoptosis is also observed in B cells that are exposed to HSV-infected fibroblasts, but BCR signaling is unaffected. Our findings suggest that the induction of apoptosis and the inhibition of TCR signal in T cells exposed to HSV-infected fibroblasts are mediated by independent mechanisms. A more complete understanding of these immune modulatory mechanisms may lead to better management of HSV infections.

Material and methods

Cells and viruses

Vero (African green monkey kidney epithelial cells), Jurkat (clone E6-1 human acute T cell leukemia), and Ramos (human Burkitt lymphoma) cell lines were obtained from the American Type Culture Collection (Manassas, VA). Human primary fibroblasts were obtained from foreskin samples. Vero cells and fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Jurkat and Ramos cells were maintained in RPMI 1640 supplemented with 10% FBS.

To isolate human $CD4^+$ cells, human blood donor white blood cell reduction filters (Pall Corporation, East Hills, NY) were obtained from Puget Sound Blood Center (Seattle, WA). Cells were extracted by alternating back flushes with phosphate-buffered saline (PBS) and 1 mM EDTA in PBS. PBMC were isolated over Ficoll-Hypaque (Fred Hutchinson Cancer Research Center, Seattle, WA). Isolated PBMC were stimulated with 5 μg/ml phytohemagglutinin in RPMI-1640 with 10% FBS for 48 h and then maintained with IL-2 in RPMI-1640 with 10% FBS. $CD4^+$ cells were positively selected to >95% purity using MACS CD4 microbeads (Miltenyi Biotec Inc., Auburn, CA) immediately prior to experiments.

HSV-1 strain F and HSV-2 strain HG52 were grown in Vero cells. Clinical isolates of HSV-1 and HSV-2 were obtained from the University of Washington diagnostic virology laboratory (Seattle, WA) and passaged twice in Vero cells to prepare viral stocks. Titers of viruses were determined in Vero cells.

Infection of T cells with HSV-1 and HSV-2

Jurkat cells were mock infected or infected with laboratory or clinical isolates of HSV-1 and HSV-2 in RPMI-1640 with 10% FBS. $CD4^+$ cells were infected with HSV-2 strain HG52 in RPMI-1640 with 10% FBS and IL-2. For each infection, viral inoculum corresponding to the MOI of 5 was added to the cells at less than 5% of total volume, and remained present throughout the incubation period. Infected cells were maintained in medium until collections at 6 h, 24 h, 48 h, and/or 72 h for detection of apoptotic markers and analysis of TCR function.

Exposure of lymphocytes to HSV-infected fibroblasts

For the exposure of Jurkat and Ramos cells to HSV-infected fibroblasts, fibroblasts were grown to confluency in flat bottom plates and then either mock infected or infected with HSV-2

HG52 at an MOI of 10 in DMEM with 2% FBS and 100 μM acyclovir (Sigma-Aldrich, St. Louis, MO). Fibroblasts were rocked during the 2-h infection period. Fibroblasts were then washed with PBS, and medium was changed to DMEM with 10% FBS and 100 μM acyclovir. After 6-h infection, fibroblasts were washed with PBS, and lymphocytes were added at 2:1 ratio (lymphocytes to fibroblasts) in RPMI-1640 with 10% FBS and 100 μM acyclovir. After 4-h incubation, lymphocytes were harvested and maintained in fresh medium with 100 μM acyclovir until each time point.

For the exposure of $CD4^+$ cells to HSV-infected fibroblasts, the experiments were conducted as above without acyclovir.

For experiments with the pan-caspase inhibitory peptide z-VAD-fmk (Calbiochem, San Diego, CA), 20 or 50 μM of inhibitor was added to Jurkat cells 30 min prior to exposure to HSV-2-infected fibroblasts and was present at the same concentration throughout the duration of the experiments.

Detection of apoptosis

Lymphocytes were collected at the stated times. Phosphatidylserine exposure on apoptotic cells was measured using annexin V conjugated with FITC (Molecular Probes, Eugene, OR) following the manufacturer's instructions. Loss of membrane integrity was measured with PI (Molecular Probes). The percentage of cells with activated caspase-3 was detected using FITC- or phycoerythrin-conjugated, affinity purified polyclonal rabbit anti-caspase-3 antibody (BD Biosciences, San Jose, CA) following permeabilization of the cells as previously described (Jerome et al., 2003). ≥10,000 events/sample were acquired using a FACSCan or LSR1 flow cytometer (BD Biosciences) and analyzed by FlowJo (Tree Star, Ashland, OR) and Cell Quest (BD Biosciences) software.

To determine the cleavage of PARP, cells were lysed in lysis buffer (20 mM Tris–HCl, pH 7.4, 10 mM EDTA, 100 mM NaCl, 1% NP-40, 0.01% sodium azide, 5 mM sodium orthovanadate, 10 mM phenylmethylsulfonyl fluoride, 10 μg/ml L-1-chlor-3-(4-tosylamido)-4-phenyl-2-butanone (TPCK), and 10 μg/ml L-1-chlor-3-(4-tosylamido)-7-amino-2-heptanon-hydrochloride (TLCK)). Nuclear and cellular debris were removed by centrifugation. Lysates were diluted 3:1 in denaturing buffer (10% SDS, 10% β-mercaptoethanol, 20% glycerol, 1% bromophenol blue, and 120 mM Tris–HCl, pH 6.8) and boiled for 5 min. Lysates from 5×10^5 cells/sample were resolved by 4–12% SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was blocked by incubating with 5% milk in PBS with 0.05% Tween-20 and probed with anti-PARP mouse monoclonal antibody (Zymed, South San Francisco, CA) overnight at 4 °C. The secondary antibody used was goat anti-mouse IgG conjugated to horseradish peroxidase (Cell Signaling, Beverly, MA). The blot was visualized by chemiluminescence with SuperSignal West Pico kit (Pierce, Rockford, IL).

Detection of calcium mobilization

Jurkat and Ramos cells were exposed to mock or HSV-2 HG52 infected fibroblasts as described above. Lymphocytes were then loaded with 2 μM of the Ca^+ indicator dye indo-1 AM (Molecular Probes) as previously described (Sloan et al., 2006). Samples were analyzed on a LSR1 flow cytometer. After obtaining a baseline, lymphocytes were stimulated for calcium mobilization with either 2 μg/ml OKT-3 (OrthoMcNeil, Redwood City, CA) for Jurkat cells or 5 μg/ml goat anti-human IgM (mu chain specific, Zymed) for Ramos cells. 400-nm to 510-nm fluorescence emission ratio versus time was plotted in Cell Quest software for analysis.

Detection of phosphorylated ERK1/2

Following exposure to mock or HSV-2 HG52 infected fibroblasts, Jurkat or Ramos cells were stimulated for 10 min at 37 °C with 10 μg/ml OKT-3 or anti-human IgM antibody respectively. Cells were then fixed, permeabilized, and stained as previously described (Sloan et al., 2006). Cells were analyzed on a FACScan flow cytometer and Cell Quest software.

Alternatively, cells were lysed following stimulation and immunoblotting was carried out as described above for the detection of PARP cleavage. Phosphorylated ERK1/2 was detected using rabbit polyclonal antibody against phospho-ERK (Thr202/Tyr204, Cell Signaling) and horseradish peroxidase conjugated goat anti-rabbit antibody (Cell Signaling). Total ERK1/2 protein was detected using rabbit polyclonal p44/42 MAP kinase antibody (Cell Signaling).

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