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Induction of SOCS-1 in HSV-1-Infected Murine Keratinocytes: A Mechanism of Inhibition of Interferon Gamma

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INDUCTION OF SOCS-1 IN HSV-1-INFECTED MURINE KERATINOCYTES:
A MECHANISM OF INHIBITION OF INTERFERON GAMMA

A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

By

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ABSTRACT


Epidermal cells such as keratinocytes are the major site of Herpes Simplex Virus Type 1 (HSV-1) replication in active primary or recurring herpes infection. In this study, a murine keratinocyte cell line (HEL-30) was shown to be refractory to IFNγ induction of an antiviral state to HSV-1 infection, while IFNγ did induce an antiviral state in a murine fibroblast cell line (L929). Particularly, IFN-γ and its peptide mimetic protected fibroblasts from viral-induced cytopathic effect while the keratinocytes were destroyed by the infection. Suppressor of cytokine synthesis-1 (SOCS-1) a negative regulator of IFNγ, was hypothesized to be responsible for the refractiveness of HEL-30 cells to IFNγ treatment. In contrast to negligible expression in the fibroblasts, HSV-1 caused the keratinocytes to express 4-fold higher levels of SOCS-1 mRNA. SOCS-1 protein was also elevated in HSV-1-infected HEL-30 cells. In agreement with these observations, activation of STAT-1α, a key protein involved in IFNγ signaling, was inhibited in HSV-1-infected HEL-30 cells while not affected in L929 cells. Additionally, HSV-1-infected HEL-30 cells showed increased activity at the SOCS-1 promoter in a luciferase assay.

Keratinocytes were responsive to the antiviral action of IFN-γ and protected from lysis by pretreatment with either a peptide antagonist of SOCS-1 or SOCS-1 small interfering RNA. An interferon-sensitive mutant of HSV-1 (dl1403) was sensitive to
IFNγ treatment in HEL-30 cells. HSV-1 *dl1403* also induced only moderate activity at the SOCS-1 promoter as compared to wild-type virus. These results suggest a role for regulation of SOCS-1 in treatment of HSV-1-induced cytopathology. Further, the actions of the SOCS-1 peptide antagonist have implications for regulation of immune responses in a number of diseases from cancer to auto-immune disorders.
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INTRODUCTION

Herpes Simplex Virus Type 1 (HSV-1) is a member of a broad family of double-stranded DNA viruses that undergo replication in the cell nucleus (Taylor et al., 2002). Family members include varicella-zoster virus (VZV) and cytomegalovirus (CMV) (Taylor et al., 2002). It is estimated that HSV-1, which persists for life in infected individuals, infects 60 to 80 percent of the people throughout the world, (Whitley et al., 2001). Primary infection commonly occurs through cells of the mucous membrane and is often asymptomatic (Favoreel et al., 2000). This is followed by uptake of virus by sensory nerve fibers and retrograde transport to the cell body of the neurons in the dorsal root or trigeminal ganglion (Stevens et al., 1971). Here, acute infection is converted to latency from which HSV-1 periodically migrates down the nerve tissue to again infect mucosal cells for overt disease (Becker, 2002).

HSV-1 infection is characterized by a strong cytokine response in infected cells, particularly the induction of type I Interferons (IFN-α and IFN-β). Infection of keratinocytes, for example, results in induction of large amounts of IFN-α and IFN-β as well as interleukins (IL) 1, 6, and β-chemokines (Sprecher et al., 1992). IFNs (Chmielarczyk et al., 1983; Decman et al., 2005b), macrophages (Kodukula et al., 1999), natural killer (NK) cells (Bukowski et al., 1986), and gamma/delta T cells (Sciammas et al., 1997) all play an important role in host innate immune response to HSV-1. Toll-like receptor (TLR) 2 is activated on the cell surface by HSV-1, while TLR-9 is activated intracellularly by viral DNA.
The latter stimulus is thought to play an important role in induction of IFN-α by HSV-1 (Gill et al., 2006).

The adaptive immune response plays an important role in confining HSV-1 and other herpesvirus infections to the latency state where CD8+ T cells (Lawman et al., 1980; Divito et al., 2006; Liu et al., 2000) and IFN-γ (Liu et al., 2001) play critical roles. Adaptive immunity is functionally connected to the innate immune system where NK cells can serve as a source of IFN-γ, which is also produced by CD4+ and CD8+ T cells (Rager-Zisman et al., 1987). IFN-γ can exert direct antiviral activity as well as induce upregulation of the antigen-presenting structures, Major Histocompatibility Class (MHC) class I and class II molecules on macrophages, dendritic cells, and keratinocytes (Albanesi et al., 2001). IFN-γ has been shown to prevent reactivation of HSV by inhibition of function of the key intermediate protein, Infected Cell Protein-0 (ICPO) (Mossman et al., 2000, 2005). Interaction of antigen-presenting cells with CD4+ T cells induces CD8+ T cells to control HSV-1 levels in mucosal lesions (Nash et al., 1980; Decman et al., 2005a).

HSV-1 has developed several mechanisms to inhibit both the innate and adaptive immune responses to infection. HSV-1 downregulation of class I MHC expression through high affinity binding of viral immediate early gene product ICP47 to the transporter associated with antigen processing (TAP) blocks IFN-γ induction of cytotoxic CD8+ T cells (Ehrlich et al., 1997). IFN-γ signaling is also inhibited by blockage of Janus kinase (JAK)/ Signal Transduction and Transcription (STAT) phosphorylation by an unknown mechanism (Yokota et al. 2001; Chee et al., 2004). ICPO is thought to enhance proteosome-dependent degradation of IFN stimulated genes (ISGs) (Sobol et al.,
A recent study suggests that HSV-1 can exert an anti-interferon effect by activation of a protein called suppressor of cytokine signaling 3 (SOCS3) (Yokota et al., 2004, 2005).

SOCS consists of a family of inducible proteins that regulate the JAK/STAT transcription system that is critical in mediation of functions of cytokines such as the IFNs (Krebs et al., 2001). These proteins share domains of homology that characterize the SOCS family, which consists of eight identified members, SOCS-1 to SOCS-7 and cytokine induced SH2 protein (CIS) (Alexander et al., 2004). All of the SOCS contain a (src-homology 2) SH2 domain and a C terminal SOCS box domain that is involved in proteosomal degradation of SOCS-associated proteins (Kile et al., 2004). SOCS-1 and SOCS-3 also contain a kinase inhibitory region (KIR) of 12 amino acids that, in conjunction with SH2, inhibits JAK tyrosine kinase activity (Waiboci et al., 2007). Thus, these SOCS-1 and SOCS-3 molecules can regulate cytokine function by proteosomal degradation of the cytokine and by inhibition of the relevant JAK activity (Kile et al., 2002).

In this study, fibroblast and keratinocyte cell lines derived from C3H mice (L929 fibroblasts and HEL-30 keratinocytes) responded differentially to IFN-γ induction of an antiviral state against HSV-1. HEL-30 keratinocytes produced large amounts of SOCS-1 mRNA and protein, while L929 cells showed minimal increase in SOCS-1 when treated with IFN-γ following infection with HSV-1. An antiviral state was induced in L929 fibroblasts but not in HEL-30 keratinocytes. The relative resistance of keratinocytes to IFN-γ therapy was due to the hyperinduction of SOCS-1 in these cells.
BACKGROUND

Clinical Aspects of Herpes Simplex Virus

Herpes Simplex Virus Type 1 (HSV-1) is a member of the alphaherpesvirus family. HSV-1 causes discontinuous, lytic epidermal lesions in the orofacial region (Taylor et al., 2002). Socio-economic status as well as race affects the demographics of HSV infection. In developing countries the infection rate is ~70-80%, while in the United States the rate is somewhat lower (Whitley et al., 2001). Infection of corneal tissues is also the main infective cause of blindness worldwide (Whitley et al., 2001). All members of the alphaherpesvirus family are neurotropic and are known to cause recurrent symptoms throughout an infected individual’s lifespan (Taylor et al., 2002). Although rarely fatal, except in individuals with compromised immunity or in neonates, HSV-1 can cause significant clinical pathology and is a source of major discomfort for infected individuals (Whitley et al., 2001).

Molecular Biology of HSV-1

HSV-1 is a large (d=150-200nm) enveloped icosahedral virus (Taylor et al., 2002) (also, refer to Figure 1). The double-stranded DNA genome codes for ≥80 genes and is organized into two covalently linked sequences (Weir, 2001). The genetic material is enclosed within the icosahedron, which is made up of repeating units of the capsid protein (Bowman et al., 2003). Between the nucleocapsid and the viral envelope lies an amorphous proteinaceous matrix known as the tegument, which contains proteins known
to enhance viral transcription, (Whitley et al., 2001). Viral Protein 16 (VP16), in particular, is a tegument protein that has been implicated in activation of viral transcription (Wu et al., 1994).

The HSV-1 genome is organized into unique long (U\textsubscript{L}) and unique short (U\textsubscript{S}) sequences that are flanked by inverted repeats (Rajcani et al., 2004). During lytic infection, viral gene expression occurs in a temporally regulated cascade. The immediate early or α-genes are transcribed first with help from VP16 (Wu et al., 1994). The immediate early genes are infected cell protein 0 (ICP0), ICP4, ICP22, ICP27 and ICP47. ICP4 and ICP22 regulate transcription of viral genes, while ICP27 and ICP47 are involved in suppressing the host response (Weir, 2001). The role of ICP0 is more complex (reviewed by Hagglund and Roizman, 2004).

Viral mutants lacking ICP0 are especially sensitive to IFN treatment (Mossman et al., 2000). Early studies suggested that ICP0 acts to induce transcription of viral genes (Cai and Shaffer, 1992; Chen and Silverstein, 1992). Further experiments indicated that ICP0 acts to increase accumulation of viral mRNA (Jordan and Shaffer, 1997). It is now accepted that ICP0 acts via binding to histone de-acetylase (HDAC) proteins (Lomonte et al., 2004). ICP0 may also function by recruiting transcription co-factors Creb Binding Protein and p300 (Melroe et al., 2006). Thus, ICP0 plays a key role in the viral life cycle by recruiting cellular co-factors and interfering with epigenetic modification of DNA. The effects of ICP0 on cellular mRNA are unknown.

The viral envelope is a host-derived lipid bilayer, in which an array of virally coded glycoproteins is embedded. These glycoproteins are essential for viral entry into host cells (Campadelli-Fiume et al., 2000). There are 11 known viral glycoproteins.
Each plays a specialized role in the sequence of events leading to fusion of the viral envelope with the host cell membrane (Campadelli-Fiúme et al., 2000). For instance, glycoprotein B (gB) mediates initial tethering to the host cell via heparin-sulfate residues on the cell surface. Subsequently, glycoprotein D (gD) binds its ligand, Herpes Virus Entry Mediator (HVE). This interaction is critical for viral entry into host cells (Huber et al., 2001). In particular, gD binds to a member of the tumor necrosis receptor family HveA. This receptor is widely expressed in a number of cell types which may explain the ability of HSV-1 to infect multiple cells types (Montgomery et al., 1996; Whitbeck et al., 1997). Significantly, HSV-1 is able to infect neurons and establish latency, a key component of viral survival. Infection of neurons is mediated by binding of gD to nectin-1 on the surface of nerve terminals. Mutations in gD that abrogate nectin-1 binding alter the ability of the virus to enter nerve cells (Manoj et al., 2004). Glycoproteins G, H, and I also play roles in viral entry.
**Figure 1: Overview of HSV-1 Macrostructure.** HSV-1 is comprised of a double-stranded DNA genome which is contained in a protein structure known as the nucleocapsid. The nucleocapsid is surrounded by a layer of proteinaceous material called the tegument. Tegument proteins have been implicated in transcription of viral genes. The virus is further contained in a host-derived lipid bi-layer which is studded with a number of virally-encoded glycoproteins. These glycoproteins, primarily gB, gC and gD, are involved in attachment to and penetration of the host cell.
**HSV-1 Infection**

HSV-1 initially infects cells at mucosal or cutaneous surfaces with preferential infection of keratinocytes. In particular, infection of keratinocytes occurs via an endocytic process (Nicola et al., 2005). Once fusion of the virus has occurred, the viral genome enters the nucleus through a nuclear pore complex (Shahin et al., 2006) (also, refer to Figure 2). During subsequent lytic infection, the virus is able to move from the initial site of infection through the underlying strata to reach the innervating neurons (Wharton et al., 1995). Studies using organotypic culture models of skin indicate that the virus is able to spread laterally and to the basal layer via microabrasions in the suprabasal cells (Visalli et al., 1997; Hukkanen et al., 1999). Viral replication in suprabasal and basal cells would permit access to nerve terminals innervating the basal layer of the epidermis. HSV-1 can infect dendritic cells and use them as a vehicle to access the local nerve bundles (Becker, 2002).

Once the virus comes into contact with neuronal cell membranes, it is able to infect these cells. Infection of neurons is dependent on binding of gD to nectin-1 on the surface of nerve cells (Manoj et al., 2004). After fusion, the virus is then transported in a retrograde manner back to the neuronal cell body in a rapid process that takes place 12-24 hours after infection (Hafezi et al., 2002). After the viral genome is transported to the cell nucleus, the virus enters a latent state (Stevens et al., 1971). The exact mechanisms that underlie latent infection are not well understood. However, some evidence points to a viral gene known as latency-associated transcript (LAT) as being essential for establishment of latency and subsequent reactivation (Perng et al., 1994).
Evasion of the Immune Response by HSV-1

In addition to exhibiting multiple cell tropisms and establishing latency, HSV-1 also uses a number of mechanisms to evade immunologic surveillance. For instance, an HSV protein, ICP47, effectively limits expression of MHC class I on the host cell surface (Ehrlich, 1997). Downregulation of MHC I by ICP47 occurs via binding to transporter associated with antigen processing (TAP) (Neumann et al., 1997). Further, ICP47 destabilizes TAP (Lacaille and Androlewicz, 1998). This has a clear deleterious effect on the ability of T cells to lyse HSV-1-infected cells. Another viral protein, ICP 34.5 is critical for evasion of the immune response as mutants lacking this protein are unable to replicate successfully, most likely due to blockage of translation (Pasieka et al., 2006). Furthermore, HSV-1 has been shown to suppress the production of pro-inflammatory cytokines by infected cells, probably due to the actions of another viral protein, VP16 (Mogensen, et al., 2004). Lysis of virions by the complement cascade has been demonstrated (Cromeans and Shore, 1981). However, HSV-1 is able to circumvent this by binding C3b in a gC-dependent manner (Kostavasili et al., 1997).

Recent evidence suggests that HSV-1 can stifle the anti-viral actions of IFNs (Yokota et al., 2001; Chee and Roizman, 2004). HSV-1 can block expression of IFN-β by recruitment of transcription factors to nuclear foci containing viral genomes (Melroe et al., 2004). The immediate early gene product, ICP0, interferes with expression of IFN-stimulated genes (Eidson et al., 2002). Recent evidence suggests that ICP0 disrupts IFN signaling via association with histone deacetylase proteins (Lomonte et al., 2004). ICP0 and ICP34.5 also act together to overcome IFN-induced blocks to viral replication (Mossman and Smiley, 2002). Additionally, ICP0 can prevent RNase L-mediated
degradation of rRNA (Sobol and Mossman, 2006). Finally, induction of suppressor of cytokine 3 (SOCS-3) has been postulated as a mechanism for viral inhibition of IFNs (Yokota et al., 2004).

Recent evidence suggests that HSV-1 can down-regulate expression of adhesion molecules of the surface of keratinocytes, thus interfering with T-cell interactions (Nikkels et al., 2004). Destruction of Langerhans cells may aid the virus by providing a longer time frame for replication in epidermal cells (Mikloska et al., 2001). Finally, HSV has been shown to be capable of degradation of cellular mRNA (Esclatine et al., 2004), inhibition of T cell activity (Sloan et al., 2006), direct lysis of T cells, disruption of NK cell function (Favoreel et al., 2000), and obstruction of antigen presentation by dendritic cells (Pollara et al., 2004).
Figure 2: **Overview of HSV-1 infection.** After initial attachment to the host cell membrane, fusion of the viral membrane with the host cell membrane occurs via the binding of viral glycoproteins to Herpes Virus Entry Mediators (HVEM) and other host cell membrane motifs. The viral genome accesses the cytoplasm and is then transported to the nucleus. Once in the nucleus, viral gene expression begins in a temporally regulated cascade. The immediate-early or α-genes are transcribed first. The products of the α-genes, primarily ICP4 and ICP0, then initiate transcription of the early (E) or β and late (L) or γ-genes (Weir, 2001).
**Epidermal Architecture and Function**

The skin is the largest organ in the body and has multiple functions. Skin serves to repel chemical and physical insults and helps to regulate loss of water and solutes to the environment. Perhaps most importantly, the skin serves as a barrier to invasion of microorganisms (reviewed by Madison, 2003). For instance, the basement membrane of the epidermis can limit spread of HSV-1 (Weeks et al., 2000). In particular, a key component of the basement membrane, laminin, can limit spread of HSV-1 in cultures of skin keratinocytes (Weeks and Friedman, 1996). Moreover, HSV-1 exhibits a tropism for the basolateral surface of cells, indicating that infection occurs at sites where the barrier has been compromised (Schelhaas et al., 2003).

The architecture of the skin is comprised of three distinct layers: epidermis, dermis and hypodermis. The uppermost layer, the epidermis, is made up largely of keratinocytes (KCs) (reviewed by Hoath and Leahy, 2003). KCs of the epidermis are organized into several layers or strata. The uppermost layer, the stratum corneum (SC), serves as the primary barrier to invading microorganisms and also wards off most chemical and physical offenses. The SC consists of enucleate cells that are rich in cytoskeletal proteins and possess a distinct lipid envelope. Cells of the SC are connected to one another by desmosomes and lie in covalently-linked lipid rafts (Madison, 2003). Directly basal to the stratum corneum is the stratum lucidum and below the stratum lucidum is the stratum granulosum. The stratum granulosum lies atop the stratum spinosum. The basal-most layer is made up of the stratum basale (SB), which is the source of epidermal stem cells (Hoath and Leahy, 2003).
Cells of each stratum are connected to and communicate with one another via a network of desmosomes (reviewed by Proksch et al., 2008). The density of desmosomes changes in relation to each stratum. For instance, the stratum spinosum contains a much higher density of desmosomes than the stratum basale (Proksch et al., 2008). Skin KCs progress through an ordered program of differentiation from the SB to the SC. In particular, KCs change expression of keratin subtypes through the differentiation process (reviewed by Bowden et al., 1987). Additionally, KCs of each stratum exhibit characteristic markers of differentiation such as expression of involucrin in the stratum spinosum (Eckhert et al., 2004). Thus, epidermal keratinocytes display distinctive traits that are indicative of the level of growth and differentiation. These markers are also helpful in determining the specific cell type(s) in isolated cultures.

**Epidermal Cell Lines**

A number of groups have isolated cultures of primary epidermal cells from mice and humans (Fusenig and Worst, 1975). However, due to limited growth potential of these cells in the absence of mesenchyme influence, epidermal cell lines were established (Hager et al., 1999). A number of transformed keratinocyte cell lines have been established and characterized. A spontaneously transformed cell line from C3H mice, HEL-30, was first isolated from neonatal mice (Franke et al., 1979). This cell line displays a polygonal morphology typical of epidermal cells. Furthermore, these cells maintain the ability to stratify in culture, forming cornified envelopes (Franke et al., 1979).

Genetic and phenotypic studies of HEL-30 cells indicated that although these cells are aneuploid, they exhibit keratinization patterns similar to cultures of primary cells
and do not express appreciable amounts of vimentin, a mesenchyme-specific marker. Keratin expression was of the K1 and K10 subtypes (Fusenig et al., 1983). Further studies of this cell line showed that these cells are capable of secretion of collagen type IV, fibronectin and lamin. Expression of these proteins results in formation of a basement membrane complete with hemidesmosomes. When cultured on collagen beds, these cells differentiate into an epithelium complete with a cornified layer (Hornung et al., 1987). Furthermore, HEL-30 cells express connexin-31, a component of gap junctions (Butterweck et al., 1994). These studies indicate that the HEL-30 cell line is a well-differentiated epidermal cell line that displays multiple characteristics of primary cells. This cell line is most similar to a cell from the stratum basale or stratum spinosum.

A keratinocyte cell line from Balb/c mice, PAM-212, was isolated by Yuspa and colleagues (1980). This cell line is immortalized via a spontaneous transformation. PAM-212 was defined as well-differentiated and keratinizing. These cells express proteins typical of epidermal keratinocytes such as pemphigus and pemphigoid (Yuspa et al., 1980). PAM-212 cells express keratins typical of basal keratinocytes, K1 and K10 (Roop et al., 1983).

Although no transformed cell line is ‘normal’, HEL-30 and PAM-212 cells can serve as useful model systems to study epidermal keratinocytes and have been used in multiple published studies. HEL-30 cells have been used in studies of arsenite exposure (Trouba et al., 2002) and allergic reactions (Van Och et al., 2005). PAM-212 has been used in studies with HSV-1 (Zak-Prelich et al., 2001).
**Immune Functions of the Epidermis**

In addition to its barrier function, the epidermis is now recognized as a participant in the immune response (reviewed by Debenedictis et al., 2001). The skin contains cells that form the skin-associated lymphoid tissue (SALT). These cells participate in the immune response. SALT produce defensins and complement factors, proteins associated with innate immunity (reviewed by Bos et al., 2001). These cells are also active in the response to infection by viruses such as HIV-1, HPV and HSV-1 (reviewed by Memar et al., 1995). SALT of the monocyte lineage, such as dendritic cells, plays a key role in the immune response to virus infection, particularly HSV-1 (Sprecher and Becker, 1989).

Once thought to be bystanders, KCs are vital contributors in the immune response. Specifically, keratinocyte-derived IL-1α functions as an inflammatory mediator. IL-1α induces expression of adhesion molecules in capillary endothelial cells (reviewed by Kupper and Groves, 1995). In addition to acting as an initiator of inflammation, KCs are capable of production of multiple cytokines that modulate the immune system. In addition to IL-1, KCs express the chemokine IL-8, IFN-α, and tumor necrosis factor alpha (TNF-α) among others (reviewed by Grone, 2002). Finally, epidermal KCs express a unique Type I IFN known as IFN-κ. This IFN has antiviral properties and stimulates IFN responsive genes (LaFleur et al., 2001). Additionally, IFN-κ can modulate cytokine release by innate immune cells (Nardelli et al., 2002).

Keratinocyte-produced TNF-α may also act in an autocrine manner. For instance, TNF-α inhibits proliferation and upregulates key mediators of immune function such as MHC class II and adhesion molecules (Detmar and Orfanos, 1990). Furthermore, TNF-α can influence the production of IL-1α by KCs (Kutsch et al., 1993). These effects have
also been observed in the PAM-212 cell line (Ansel et al., 1990). Finally, TNF-α may be critical for inhibition of virus as knockout mice show decreased survival when infected with HSV-1 (Minagawa et al., 2004).

Keratinocytes also participate in activation of T-cells (reviewed by Nickoloff et al., 1995). In particular, KCs produce IL-18 which promotes expression of IFN-γ by T-cells (Companjen et al., 2000). HEL-30 cells also produce IL-18 in appreciable amounts (Van Och et al., 2005). Treatment of KCs with IFN-γ increases expression of MHC class II molecules (Cunningham and Noble, 1989). KC class II molecules are fully capable of loading peptide for presentation to T-cells (Albanesi et al., 1998). Thus, KCs may act as antigen presenting cells to CD4+ T cells.

Stimulation of KCs with T-helper 1 (T_H1) cytokines such as IFN-γ increases expression of chemokines (Albanesi et al., 2001). This suggests that KCs can create an inflammatory environment conducive to migration of increased numbers of T_H1 cells. Expression of T_H1 cytokines is associated with increased resistance to HSV-1 (Koelle et al., 1998). Furthermore, expression of ICAM-1 by KCs also enhances proliferation of T cells (Haw, 1995). These observations suggest that keratinocytes may influence the inflammatory environment through stimulation of immune cells, secretion of chemokines and self-modulation of gene expression.

**Cytokine Production by HSV-1-Infected Epidermal Cells**

Recent studies have demonstrated that keratinocytes are capable of producing a panel of pro-inflammatory cytokines in response to virus infection and other insults (reviewed by Hukkanen, 2002). For instance, infection of keratinocytes with Vaccinia virus results in expression of immunoregulatory cytokines IL-10 and IL-13 (Liu et al.,
In a study of HSV-1 infection of primary keratinocytes from Balb/C and C57Bl/6 mice, Specher and Becker (1992) observed production of IL-1β, TNF-α and IL-6. Conversely, production of IL-1α is inhibited by HSV-1 infection (Enk et al, 1991). Treatment of keratinocyte cell lines with TNF-α has been shown to increase production of IL-1α and the IL-1 receptor (Kutsch et al, 1993) suggesting that TNF-α production by infected keratinocytes may act to amplify the inflammatory response by upregulating IL-1α production. TNF-α also exerts a protective effect in HSV-1-infected mice (Rossol-Voth et al., 1991). On the other hand, PAM-212 cells seem to produce only IL-10 in response to HSV-1 infection and no IL-1α or TNF-α (Zak-Prelich et al, 2001). This may benefit the virus because IL-10 can inhibit expression of IFNs (Ito et al., 1999).

Studies using human cell lines indicate that HSV-1 infected keratinocytes are capable of producing IFN-α (Schnipper et al, 1984), IFN-β (Torseth et al, 1987), IL-10, IL-6 and IL-12 (Mikloska et al, 1998). This may represent species-specific differences in the response to HSV-1 infection.

Other types of epithelial cells, such as corneal epithelium, also produce pro-inflammatory cytokines in response to infection with HSV-1 (Li et al., 2005). In addition, corneal epithelium expresses mRNA for chemokines (Cook et al., 2004). This suggests distinctive patterns of cytokine production by different cell lines and perhaps differences between primary and transformed cells. In addition, use of different viral strains in these studies may affect the results.
Dermal Architecture and Function

The layer directly below the epidermis is known as the dermis. The dermis is composed mainly of fibroblasts and collagen bundles. The dermis is further sub-divided into papillary and reticular dermis. The primary functions of the dermis are to provide structural support and necessary metabolites needed for epidermal maintenance and growth (reviewed by Rinn et al., 2008). The hypodermis is located underneath the dermis and houses sweat glands and touch receptors.

Skin KCs respond to a number of extra-cellular stimuli such as calcium and cytokines (Proksch et al., 2007). Perhaps most importantly, epidermal KCs communicate with and are regulated by the cells of the dermis (reviewed by Mueller and Fusenig, 2002). Studies using transformed epidermal cell lines indicated that co-culturing with mesenchyme restored expression of differentiation specific or ‘suprabasal’ keratins such as K1 and K10 in the keratinocytes (Breitkreutz et al., 1986).

Basement membrane formation by epidermal cells is also influenced by mesenchyme, presumably via diffusible factors (Bohnert et al., 1986; Smola et al., 1998). The importance of mesenchyme-derived diffusible factors in regulation of keratinocytes growth and differentiation was later confirmed in experiments using primary epidermal cells grown on collagen gels or isolated dermal sheets. The epidermal cells grown on isolated dermal sheets showed increased proliferation and differentiation, expression of K1 and K10, and morphology similar to that of in vivo skin (Mackenzie et al., 1993).

The discovery of keratinocyte growth factor (KGF) added further support to the idea that a fibroblast-produced cytokine may be able to influence epidermal growth and differentiation. In fact, the production of KGF by fibroblasts is most likely influenced by
the keratinocyte itself (Smola et al., 1993). Production of IL-1α by keratinocytes acts to stimulate production of growth factors by fibroblasts (Maas-Szabowski and Fusenig, 1996). Specifically, production of KGF is blocked in fibroblasts co-cultured with keratinocytes if antibodies to IL-1α are present in the culture medium. Conversely, keratinocyte growth and differentiation is slowed in co-cultures with antibodies to KGF in the medium (Maas-Szabowski et al., 1999; Maas-Szabowski et al., 2000). In addition, keratinocytes-derived IL-1 can modulate production of IL-6 by fibroblasts (Boxman et al., 1993). Further studies using fibroblasts isolated from papillary or reticular dermis indicated that papillary fibroblasts are more effective at supporting KCs than their reticular counterparts, indicating site-specific signaling mechanisms (Sorrell et al., 2004). Thus, the current paradigm of fibroblast-keratinocyte interaction is a double paracrine model in which bi-lateral communication regulates gene expression in each cell type. This inter-cellular communication is perhaps most important in the generation of an immune response to invading micro-organisms.

**Immune Functions of the Dermis**

Besides its role in structural and metabolic support of the epidermis, the dermis plays a role in the immune response. Multiple dermal cells participate in immune function. Primary among these are cells of monocyte origin such as dendritic cells and dermal macrophages. These cells are critical for antigen presentation (reviewed by Mathers and Larregina, 2006). Further, mast cells in the dermis are active in allergic responses but also interact with cells of innate and adaptive immunity (reviewed by Rao and Brown, 2008).
Fibroblasts also have immune functions. In particular, fibroblasts secrete pro-inflammatory cytokines that mediate extra-vasation of leukocytes (McGettrick et al., 2009). Recent evidence suggests that chemokine expression by fibroblasts aids in dendritic cell migration to lymph nodes (Ouwehand et al., 2008). Conversely, expression of macrophage inhibitory factor by fibroblasts may slow migration of macrophages (Kim et al., 2008). Maturation of dendritic cells is dependent on interactions with dermal fibroblasts (Saalbach et al., 2007). This was dependent on expression of TNF-α, suggesting that fibroblasts can secrete this cytokine. Fibroblasts may also modulate T-cell proliferation through expression of IDO in an IFN-γ-dependent manner (Haniffa et al., 2007). On the other hand, interactions of fibroblast with T-regulatory cells may dampen inflammation and suppress proliferation of T_H cells (Clark and Kupper, 2007). Finally, as discussed above, fibroblasts play a central role in wound healing. Thus, dermal cells contribute to the immune response via expression of pro-inflammatory cytokines and modulation of leukocytes.

_Murine Immune Response to HSV_

Different antigenic specificities are seen in mice of differing genetic backgrounds. Early experiments using C57BL/6 mice indicated that cytotoxic T-Lymphocyte (CTL) response was directed mostly toward antigens derived from gC (Marlin et al., 1985) as CTLs were capable of lysing transfected cells that expressed gC only. Cells expressing gB or gD were not affected (Witmer et al., 1990). However, a subsequent study showed that CTL from C57BL/6 mice could recognize gB antigens (Bonneau et al., 1993). Further research showed that up to 90% of CTL derived from C57BL/6 mice were specific for one gB-derived peptide (Wallace et al., 1999). In contrast, C3H mice
produce neutralizing antibodies against gC and gD (Mester et al., 1991) while Balb/C mice generate T cell responses to gD-derived antigens. Moreover, Balb/C-derived T cells have an appreciable response to only one gD peptide (Yamashita and Heber-Katz, 1989).

Conversely, when BALB/c mice were infected with HSV, at least 17 different gD peptides generated a T cell response (Yamashita and Heber-Hatz, 1989). These results suggest that the immune response to HSV infection is significantly different from that generated by immunization with peptides. Since previous studies using C57BL/6 mice used transfected cells (Witmer et al., 1990) or adenovirus vectors (Bonneau et al., 1993) as vehicles for expression of various HSV glycoproteins, it is not surprising that different results would be obtained from infected animals. Later studies showed CD4+ T cells in the initial influx of cells into draining lymph nodes (Hayashi et al., 1991). Subsequently, CD4+ T cells were shown to be the main effectors of viral clearance in immunized mice (Manickan et al., 1995).

Taken together, the studies discussed above suggest that CD4+ T cells mainly control the murine response to HSV infection. This is not surprising given the observation that HSV-1 interferes with presentation of antigen on MHC class I molecules (Lacaille and Androlewicz, 1998). CTL responses would be insufficient for viral clearance. Since IFN-γ can upregulate MHC I expression (Mikloska et al., 1996), IFN-γ is critical in control of HSV-1.
C3H Mouse Response to HSV

The host response to HSV infection is varied and complex. Mice represent the most used and extensively studied experimental system. Reasons for this include existence of inbred mouse strains, availability of immunological reagents, and ethical concerns in using human subjects. As such, murine studies are used to model the human response.

The C3H mouse has been shown to be partially resistant to infection by HSV-1. A robust NK cell response has been postulated as one mechanism for this resistance (Armerding and Rossiter, 1981). On the other hand, Type I IFN production has been proposed as a mechanism for resistance (Chmielarczyk et al., 1985). High levels of Type I IFN are present in exudates as soon as 2-4 hours after infection (Engler et al., 1982). Evidence suggesting that IFN-γ can stimulate NK cell activity helps to reconcile the differences in those two studies (Kohl, 1983). In contrast, Balb/c mice are not known to be particularly resistant and produce low levels of Type I IFN but show strong NK cell activity. Early in HSV-1 infection a large influx of neutrophils cells is observed. These cells act to confine the infection to the initial site (Watanabe et al., 1999).

NKT cells have been observed at the site of peritoneal infection as early as 4 hpi (Frye and Bigley, unpublished observations). Natural Killer (NK) cells can be seen in peritoneal exudates as early as 24 hpi. These cells exhibit cytotoxic activity towards autologous HSV-1 infected cells (Armerding et al., 1981). Cytotoxic activity declines at day 4 p.i. but returns vigorously on day 6 p.i. The second burst of cytotoxic activity is likely caused by T lymphocytes infiltrating the area.
A T cell response is seen as early as three days and peaks at 1 week p.i. The primary response is comparatively weak (Lawman et al., 1980). T cells were undetectable after 2 weeks. In studies using corneal injection T cell numbers peaked at day 6 p.i., indicating a slight lag in the T cell response in this model (Hirakawa et al., 1985). Both of these studies used spleen cells to measure cytotoxicity. Although this method certainly provides information about the host response to infection, the results can only be applied indirectly to the cell populations that are actually effective at the infection site.

Studies using the ear pinna models have demonstrated increased proliferation of cytotoxic T cell in the draining lymph nodes (DLN) at 4-6 days p.i. (Nash et al., 1980). A large mononuclear cell infiltrate was observed in the pinna by day 3. Likewise, in recurrent infection of the cornea, a large influx of granulocytes is observed after reactivation (Shimeld et al., 1996).

An early study using an epidermal infection observed lymphoproliferation in the draining lymph nodes 3 days p.i, reaching a maximum at 6 days p.i. (Maingay et al., 1989). Proliferating populations consisted of mainly T cells of the T\textsubscript{H1} subtype. Antibody titres were undetectable prior to day 6 p.i. and peaked day 9 p.i., indicating that B cells do not play a major role in the primary response. Later the T\textsubscript{H1} cells were shown to produce IL-2, a known T\textsubscript{H1} cytokine (Prymowicz et al., 1985). \(\gamma\delta\) T cells can be seen as early as 2 days p.i., while \(\alpha\beta\)-T cells are scarcely seen at this time point (Monma et al., 1996).

These results indicate that the early response (>72 hours) to HSV-1 infection is dominated by NK cells and granulocytes, at least in the i.p. model. Expression of IFN-\(\gamma\) seems to be critical for priming of an efficient immune response that is primarily T\textsubscript{H1}
based. Other T cell subtypes such as γδ T cells and NKT cells may play a role in early control of the infection. Studies done using the ear pinna model and gingival infection seem to confirm these results. Observations of DLN after infection indicate that T cells are actively proliferating by day 3 p.i. and begin to enter the tissues at day 4 p.i. Presumably, the infection is initially managed by cells of the innate immune system. This is followed by viral clearance by cytotoxic T cells.

**Significance of IFN-γ in HSV-1 Infection**

IFN-γ is a pleiotropic cytokine with multiple actions, including establishment of an anti-viral state in target cells. The critical role of IFN-γ in resistance to HSV-1 has been demonstrated in experiments with knockout mice. Mice lacking the gene for IFN-γ show decreased survival when infected with HSV-1 (Minami et al., 2002). In addition, an IFN-γ-associated transcription factor, STAT-1, is able to regulate viral latency by binding to the LAT promoter (Kriesel et al., 2004). The actions of IFN-γ on keratinocytes are specific and targeted.

For instance, IFN-γ can reverse viral-mediated downregulation of MHC class I molecules in HSV-1-infected KCs (Mikloska et al., 1996). Increased expression of MHC I molecules can boost cytotoxic activity of CD8+ T cells. In addition, IFN-γ stimulates increased expression of MHC class II which improves the cytotoxic actions of CD4+ T cells (Mikloska and Cunningham, 1998). Furthermore, IFN-γ treatment increases expression of tight junction components and down-regulates cell growth and differentiation programmes (Banno et al., 2003). This would hinder the ability of the virus to access neighboring cells and perhaps stall viral replication. This may provide a
lengthened time frame for the adaptive immune system to respond. Thus, IFN-γ is important for enhanced elimination of virally-infected epidermal cells.

TNF-α can protect mice in vivo from lethal infection with HSV-1 (Rossol-Voth et al., 1991). IFN-γ synergizes with TNF-α to inhibit replication of HSV-1 (Feduchi et al., 1989). The mechanism of viral inhibition is hypothesized to be increased expression of IFN-β (Feduchi and Carrasco, 1991). Increased levels of IFN-β would result in augmented resistance to virus infection. Conversely, some evidence suggests that inhibition of HSV-2 by TNF-α and IFN-γ is mediated by an enzyme involved in tryptophan metabolism (Adams et al., 2004). These seemingly contrary findings may be explained by the fact that the experiments with HSV-2 used an astrocytoma model system rather than epithelial cells. In addition, extremely low MOIs were used in these experiments.

In addition to synergizing with TNF-α, IFN-γ also can synergize with IFN-α/β to inhibit replication of HSV-1 (Sainz and Halford, 2002). Treatment of cells with IFN-β and IFN-γ inhibits viral replication and mRNA accumulation to a greater extent than treatment with either IFN alone (Pierce et al., 2005). Moreover, IFN α/β treatment combined with IFN-γ suppresses infection in mice with no adaptive immune response (Vollstedt et al., 2004). IFN-β/γ-induced inhibition of cytomegalovirus, another member of the herpesvirus family, has also been observed (Sainz et al., 2005). Finally, IFN-γ can enhance production of IFNκ in epidermal KCs (LaFleur et al., 2001).

These results suggest that IFN-γ can establish an antiviral state either alone or in tandem with other cytokines. These studies also suggest that IFN-γ plays a critical role in the immune response to acute infection with HSV-1, most likely by potentiating and
amplifying the actions of the Type I IFNs and other cytokines involved in innate immunity. Perhaps more importantly, IFN-γ production by CD8\(^+\) T cells is essential for maintaining virus latency (reviewed by Khanna et al., 2004).

**Overview of IFN-γ Signaling**

Biochemical and genetic studies have revealed the major signal transduction pathway activated by IFN-γ (reviewed by Briscoe et al., 1996) (also refer to Figure 3). The receptor for IFN-γ is comprised of two subunits, IFNGR1 and IFNGR2. The Janus kinases JAK1 and JAK 2 are bound to IFNGR1 and IFNGR2, respectively. Binding of IFN-γ causes oligomerization of the receptor chains and activation of the JAK proteins (Briscoe et al., 1996). Activated JAK proteins then phosphorylate key tyrosine residues on the receptors. These phosphotyrosines act as docking sites for the transcription factor STAT-1. STAT-1 is then phosphorylated on Tyr\(^{701}\), homodimerizes and translocates to the nucleus. Once in the nucleus, pSTAT-1 binds to DNA and initiates transcription of IFN-γ–responsive genes (reviewed by van-Boxel-Dezaire and Stark, 2007). More recent studies indicate that a macromolecular complex comprised IFNGR1, pSTAT-1 and IFN-γ itself are endocytosed and subsequently shuttled to the nucleus (Larkin, J 3\(^{rd}\) et al., 2000). Once in the nucleus, this complex is able to transactivate IFN target genes (Ahmed and Johnson, 2006). The JAK/STAT pathway represents the canonical and primary pathway of IFN-γ signaling. It should be noted that alternate pathways have been elucidated (reviewed by Subramaniam et al., 2001). In addition, signaling that is independent of STAT-1 has also been described (reviewed by Ramana et al., 2002).

IFN-γ signaling is tightly regulated at a number of levels (reviewed by Wormald and Hilton, 2004). For instance, SH2-containing phosphatase (SHP2) inhibits activation
of STAT-1 via binding to IFNGR1 (You et al., 1999). Protein inhibitor of activated Stat-1 (PIAS) prevents activation of transcription via sequestration of STAT-1 (Liu et al., 1998). However, the most potent inhibitor of IFN-γ is Suppressor of Cytokine Signaling 1 (SOCS-1).
Figure 3: IFN-γ signaling via the JAK-STAT pathway. Binding of IFN-γ to its receptor induces auto-activation of the protein kinases JAK1 and JAK2. Multiple tyrosine residues on the receptor are phosphorylated. Phosphorylated residues on JAK2 serve as docking sites for the transcription factor Stat-1α. Through the actions of JAK2, Stat-1α is phosphorylated on key tyrosine residues. Stat-1α then homo-dimerizes and translocates to the nucleus where it binds to DNA regions containing gamma activated sequence (GAS) elements. Stat-1 can then initiate transcription of gamma-responsive genes such as oligoadenylate synthase (OAS) and SOCS-1. SOCS-1 is a critical negative feedback regulator of IFN-γ signaling.
SOCS-1 Structure and Regulation

SOCS-1 is a member of a protein family involved in regulation of cytokine responses (reviewed by Alexander and Hilton, 2004). There are eight members of the SOCS family, CIS and SOCS1-7. These proteins share common structural components including a central SH2 domain and a C-terminal domain known as the SOCS box (refer to Figure 4).

SOCS-1 was initially identified as an inhibitor of IL-6-mediated macrophage differentiation (Starr et al., 1997). Expression of SOCS-1 is increased in response to cytokine signaling (Naka et al., 1997). In particular, IFN-γ induces expression of SOCS-1 (Sakamoto et al., 2000). Further study of SOCS-1 revealed that it binds to JAK proteins and inhibits their kinase activity (Endo et al., 1997). Specifically, SOCS-1 binds to the catalytic domain of JAK2, preventing activation (Yasukawa et al., 1999). More recent evidence suggests that SOCS-1 antagonizes JAK activation only after binding to a key phosphotyrosine on Interferon Gamma Receptor 2 (IFGNR2) (Qing et al., 2005). Thus, SOCS-1 acts in a classical negative feedback loop. Experiments with SOCS-1−/− mice demonstrated the absolutely essential nature of SOCS-1. Knockout mice die within three weeks of birth with fatty degeneration of liver and massive inflammatory infiltrates of major organs (Starr et al., 1998). In addition, SOCS-1 protects oligodendrocytes from IFN-γ-induced cell death (Balabanov et al., 2006).

SOCS-1 expression is regulated at a number of levels. In response to IFN-γ, SOCS-1 accumulates slowly over a period of 2-4 hours (Wormald et al., 2006). Expression of SOCS-1 mRNA is antagonized at the level of translation initiation (Gregorieff et al., 2000). This is most likely due to the presence of an open reading frame (ORF) upstream of the SOCS-1 ORF. Assembly of ribosomes at the upstream ORF may
act to inhibit translation from the SOCS-1 ORF (Schluter et al., 1999). Finally, Pim kinase may act to stabilize SOCS-1 via phosphorylation (Chen et al., 2001).

**Regulatory Functions of SOCS-1**

Although SOCS-1 was first identified as an inhibitor of IL-6, subsequent experiments demonstrated that SOCS-1 and SOCS-3 inhibit IFN-γ signaling as well (Song and Shuai, 1998). Importantly, SOCS-1 inhibition of IFN signaling is much more robust than that of SOCS-3. Further experiments with SOCS-1-null mice indicated that SOCS-1 is critical for inhibition of IFN-γ signaling. Treatment of these mice with neutralizing antibodies to IFN-γ was able to prevent the lethal phenotype (Alexander et al., 1999). Moreover SOCS-1-null mice showed increased activation of STAT-1 (Brysha et al., 2001).

The regulatory actions of SOCS-1 are not limited to inhibition of JAK activation. All members of the SOCS family contain a domain known as the SOCS box. The SOCS box is shared by over 30 protein families. It functions as a link to the proteosomal degradation pathway via an E3 ligase activity (Vuong et al., 2004). Thus SOCS-1 is able to mediate degradation of JAK2. Notably, degradation of JAK2 is reliant on strong binding of SOCS-1 to activated JAK (Kamizono et al., 2001). Specifically, degradation of activated JAK2 is contingent on binding of SOCS-1 to a phosphotyrosine residue in the catalytic loop of JAK2 (Ungureanu et al., 2002). This suggests that SOCS-1 not only attenuates but terminates the signaling actions of activated JAK2. SOCS-1 seems to be solely responsible for JAK2 degradation since SOCS-3 is unable to mediate degradation of JAK2 (Ungureanu et al., 2002). Furthermore, fusion of the SOCS box of SOCS-3 to SOCS-1 is not able to recapitulate this function (Kamizono et al., 2001). The importance
of SOCS-1 in regulation of IFN-γ signaling was demonstrated in mice that express SOCS-1 lacking the SOCS box. These mice are hypersensitive to IFN-γ and die of inflammatory disease when treated with IFN-γ (Zhang et al., 2001). Thus, SOCS-1 is a primary regulator of IFN-γ signaling.

SOCS-1 is also implicated in regulation of Type I IFNs. For instance, SOCS-1 can inhibit Type I IFN activity (Fenner et al, 2006) and can dampen their anti-viral actions (Vlotides et al., 2004). This is most likely mediated by Toll-like Receptor (TLR) signaling since SOCS-1 is induced by CpG-DNA in antigen presenting cells (Dalpke et al., 2001). Indeed, current evidence suggests that SOCS-1 can regulate TLR signaling via degradation of a key TLR-associated protein (Mansell et al., 2006). However, contrary evidence exists and this is an area of intense effort in the field (Prele et al., 2008).

SOCS-1 can hamper macrophage function (O’Keefe et al., 2001; Wesemann et al., 2002) and reduce the pro-inflammatory effect of IL-10 (Ding et al., 2003). Further, SOCS-1 can inhibit activation of keratinocytes (Albanesi et al., 2002; Federici et al., 2002). SOCS-1 is suggested to have a number of other functions from modulation of fibroblast growth factor (Ben-Zvi et al., 2005) to a possible role as a tumor suppressor (Rottapel et al., 2002). Given the pivotal role of SOCS-1 in cytokine signaling, IFN-γ in particular, it represents a logical target for viral interference. Immunotherapeutic development should seek approaches to modulate SOCS-1 in order to enhance the immune response.
Figure 4: Structure of the SOCS Protein Family. The SOCS family is comprised of 8 members, CIS and SOCS 1-7 (percentages reflect amino acid homology). Each member of the family shares common structural components. All family members contain a central SH2 domain which mediates binding to phosphotyrosine residues. Additionally, each member contains a C-terminal SOCS box. The SOCS box has been implicated in mediation of protein degradation via the ubiquitin-proteosome pathway. SOCS-1 and SOCS-3 also contain a 12 amino acid motif known as the kinase inhibitory region (KIR).

**Use of Peptide Mimetics to Modulate the Immune Response**

Peptide mimetics have been used to modulate cell function for many years. For instance, the use of a tripeptide to mimic the natural ligand of cell adhesion molecules reduced liver pathology in concanavalin A-treated mice (Bruck et al., 1997). The use of molecular mimicry to induce antibody production has also been described (Cunto-Ametsy et al., 2001). Further, peptides have been used as candidate vaccines (Partidos et al., 2001). Similarly, peptide antagonists of HveA can block binding of HSV-1gD (Sarrias et al., 1999). This may have implications for prophylactic treatment against viral infection. Finally, treatment of cancer with a peptide mimetic of the anti-angiogenic protein thrombospondin-1 has also been described (Reiher et al., 2002).

Peptide mimetics of several cytokines have been studied. For instance, peptides representative of TGF-β show neuroprotective effects (Zhang et al., 2005). In addition, peptide mimetics of IFN-γ function in a manner similar to the full-length protein (Szente et al., 1994). In particular, IFN-γ peptides are able to protect cells from Vaccinia virus-induced cell pathology. Anti-viral effects of the peptide are functional even in the presence of a protein that binds to and sequesters IFN-γ (Ahmed et al., 2005). Furthermore, IFN-γ peptides can prevent lethal infection in a murine model of Endomyocardial Virus (EMCV) infection (Mujtaba et al., 2006). In fact, the peptide mimetic may be superior for induction of anti-viral pathways (Fulcher et al., 2008). Thus, cytokine peptide mimetics have applications as antiviral compounds. The use of peptides to modulate signaling pathways also represents an approach for development of immunotherapeutics.
Modulation of IFN-γ in particular has intriguing uses. A peptide inhibitor of JAK2 has been characterized (Flowers et al., 2004). This peptide, known as Tyrosine Kinase Inhibitory Peptide (Tkip), can suppress IL-6-mediated activation of STAT-3 (Flowers et al., 2005). Tkip has striking effects \textit{in vivo}. For instance, treatment of mice with Tkip is able to prevent experimental allergic encephalomyelitis (Mujtaba et al., 2005). Furthermore, Tkip protects mice from lethal poxvirus infection (Ahmed et al., 2009). The success of Tkip suggests a mechanism for inhibition of SOCS-1. Indeed, a peptide inhibitor of SOCS-1 has been characterized. This peptide is able to potentiate IFN-γ signaling and anti-viral actions (Waiboci et al., 2007). Thus, use of peptides to modify the actions of IFN-γ may be useful in treatment of viral infections.

**Significance of this study:**

While most studies on HSV-1 infection have focused on the T cell or adaptive response, the innate immune response to HSV-1 has been largely ignored. One area of the field that has received little attention is the early innate immune response to infection of epidermal surfaces. Current evidence suggests that the efficacy of the adaptive immune response is dependent upon efficient priming by the innate response. Because tissue cells at the site of infection, such as keratinocytes, were once assumed to play little if any role in the immune response, previous studies to investigate the early host response to HSV-1 used an intraperitoneal model.

More recently, it has been shown that keratinocytes grown in monolayer cell culture are capable of producing a group of pro-inflammatory cytokines such as IL-1α, IFN-α, TNF-α, IL-10, IL-12 and IL-18 (Grone, 2002). The essential nature of cytokines produced by KCs is reflected in a number of studies. Production of chemokines by KCs
is vital for migration of leukocytes (Kupper and Groves, 1995; Albanesi et al., 2001). Production of TNF-α by KCs may act to potentiate chemokine production (Kutsch et al., 1993). Antigen presentation by keratinocytes may represent a critical method for elimination of virally-infected cells (Albanesi et al., 1998). Increased expression of ICAM-1 enhances proliferation of T cells (Haw et al., 1995). KC-derived IL-18 boosts production of IFN-γ by T cells (Companjen et al., 2000). Thus, interactions between cells of the immune system and KCs are crucial for a productive immune response.

IFN-γ produced by T\textsubscript{H}1 cells exerts a marked effect on the production of cytokines by keratinocytes. IFN-γ increases production of chemokines by KCs (Albanesi et al., 2001). Furthermore, IFN-γ can improve expression of IFNκ (LaFleur et al., 2001). IFN-γ up-regulates expression of MHC class II molecules on KCs (Mikloska and Cunningham, 1998). Synergism between IFN-γ and TNF-α or IFN-β augments anti-viral pathways (Feduchi et al., 1989; Sainz and Halford, 2002). Finally, IFN-γ modulates expression of cell growth and differentiation programmes in KCs (Banno et al., 2003).

Given the key role of IFN-γ in resistance to HSV-1 (Minami et al., 2002) and its ability to affect KCs, studies IFN-γ treatment of HSV-1-infected skin cells are important in our understanding of host-virus interactions. These studies are increasingly important in light of evidence suggesting that HSV-1 can inhibit IFNs (Yokota et al., 2001; Chee and Roizmann, 2004). These studies demonstrated that HSV-1 blocks the JAK-STAT pathway. However, the proposed mechanism, induction of SOCS-3 (Yokota et al, 2004), is unsatisfactory when the dissimilar impact of SOCS-1 and SOCS-3 on IFN-γ signaling is taken into consideration (Ungureanu et al., 2002). It is more likely that SOCS-1 is responsible for inhibition of IFN-γ. Thus, a re-examination of the inhibition of IFN-γ...
signaling by HSV-1 needs to be undertaken. Further, use of existing immunotherapeutics to reverse viral-mediated inhibition may reveal strategies for treatment of HSV-1.
MATERIALS AND METHODS

Cell Lines and Cell Culture: HEL-30 keratinocytes (a kind gift of Dr. D. Germolec, National Institutes of Health), L929 fibroblasts (CCL-1, ATCC, Manassas, VA) (Sanford et al., 1948), PAM-212 keratinocytes (Joanna Anders, NIH/NCI, Bethesda, MD), A.2R.1 (TIB-86, ATCC), RAW 264.7 macrophages (Dr. Julian Gomez-Cambronero, Wright State University, Dayton, OH) and Vero cells (CCL-81, ATCC) were cultured in DMEM (Mediatech, Manassas, VA) supplemented with 10% bovine calf serum (BCS) (Hyclone, Logan, UT). U2-OS cells (HTB-96, ATCC) were cultured in McCoy’s 5A (ATCC) supplemented with 10% fetal bovine serum (FBS) (Hyclone). Cells were plated into 100 mm² tissue culture dishes and incubated at 37°C, 95% air/5% CO₂ in a humidified incubator. Cells were sub-cultured by disaggregation with 0.25% trypsin (Sigma-Aldrich, St. Louis, MO) in 1x Hanks Balanced Salt Solution (1x HBSS) (Mediatech).

Virus Strains and Propagation: HSV-1 (syn 17⁺) (Dr. Nancy Sawtell, Children’s Hospital Medical Center, Cincinnati, OH) was routinely passaged and titrated in Vero cells (He et al, 1999). The ICP0⁻ mutant (dll1403) of syn 17⁺ was a kind gift of Dr. Rick Thompson (University of Cincinnati, Cincinnati, OH). This virus was originally developed by Dr. Nigel Stow (Medical Research Council, Glasgow, Scotland) (Stow and Stow, 1986). HSV-1 dll1403 was passaged and titred in U2-OS cells. HSV-1 (KOS) and its mutant ICPO⁻-GFP were kindly provided by Dr. William Halford (Montana State University, Bozeman, MT) (Halford et al., 2006). HSV-1 (KOS) was passaged and titrated in Vero cells.
**Peptides:** The amino acid sequences for the peptide mimetics used in this study shown in Table I. The peptides were provided by Dr. Howard Johnson (University of Florida, Gainsville, FL). Peptides were synthesized on an Applied Biosystems 9050 automated peptide synthesizer using conventional fluorenylmethyloxycarbonyl chemistry as previously described (Szente et al., 1996). The addition of a lipophilic group (palmitoylllysine) to the N terminus of the synthetic peptide was performed as a last step, using semiautomated protocol (Thiam et al., 1999). Peptides were characterized by mass spectrometry and were purified by HPLC. All peptides were dissolved in DMSO at a concentration of 10 mg/mL. Peptides were diluted in cell culture medium prior to addition to cells.

**Table I. Amino acid sequences of the peptides used in this study**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>JAK-m</td>
<td>1001 LPQDKEAAKVKEP</td>
</tr>
<tr>
<td>pJAK-2</td>
<td>1001 LPQDKE YKVKEP</td>
</tr>
<tr>
<td>T-Kip</td>
<td>WLVFFVIFYFFR</td>
</tr>
<tr>
<td>T-Kip ctrl</td>
<td>WLVAAVIAYFAA</td>
</tr>
<tr>
<td>Mu IFN-γ95-106</td>
<td>95 AKFEVNNPQVQR</td>
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<tr>
<td>Mu IFN-γ95-133</td>
<td>95 AKFEVNNPQVQRQAFNELIRVVHQLLPESSL</td>
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*a Peptides were synthesized as described previously. Murine IFN-sequence is derived from the mature form. Lipophilic group and biotinylated modifications were added to the N terminus of the peptide. Tyrosines targeted for phosphorylation are indicated in bold. The bold, underlined text denotes the phosphotyrosine moiety. The JAK2 WT sequence is the same for both mice and humans.
**Cytopathic Effect Inhibition Assay:** This assay is a modification of an assay originally developed by Pestka and colleagues (Rubinstein et al., 1981). L929 fibroblasts, HEL-30 keratinocytes, PAM-212 keratinocytes, A.2R.1 fibroblasts, or RAW 264.7 macrophages were cultured and counted in a hemacytometer, added at densities of 2.0 x 10^4 to 3.0 x 10^4 to each well of a 96-well cell culture plate and incubated overnight. The following day, recombinant murine interferon gamma (IFN-γ) (Peprotech, Rocky Hill, NJ), peptide mimetics or peptide mimetics with IFN-γ were added to the cultures at the indicated concentrations and incubated for 24H. At 100% confluence, culture medium was aspirated, cells were rinsed with DMEM/2% FBS (maintenance medium) and HSV-1 diluted in maintenance medium was added at an MOI of 0.1. Virus was allowed to adsorb for 2 hours and plates were rinsed with maintenance medium. Fresh maintenance medium was added and then plates were incubated for 48 hours. Two days post-infection (p.i.), medium was aspirated; cells were washed twice with 1x HBSS and then fixed by addition of 10% formalin. Fixative was removed and then cell layers were stained with 0.05% crystal violet. Plates were rinsed with dH_2O and then dried overnight. Plates were scanned on an HP ScanJet 5300C or photographed using a Fuji LAS-300 CCD camera (Fujifilm USA, Burbank, CA). Densitometry measurements of each well were computed using Multi-gauge software (FujiFilm USA, Burbank, CA) or NIH Image-J.

**HSV-1 infection of Monolayer Cultures:** Cells were seeded into 35 mm culture dishes at a density of 1 x 10^4 cells/cm^2 and then allowed to grow to ~75% confluence. Culture medium was aspirated and then monolayers were washed with 1x PBS. HSV-1 diluted in maintenance medium was added to at the indicated MOI and then cell cultures were incubated for 2 hours at 37°C. Infection medium was removed and replaced with DMEM
containing 10% CS. For Western blotting, cell lysates were prepared at the indicated hours post-infection (hpi).

**RNA isolation and Quantitation:** RNA was collected from cells at specified times after infection. Total RNA was isolated by using RNeasy mini kits (Qiagen Inc., Valencia, CA) according to the manufacturers’ instructions. Samples were eluted twice in a volume of 20 µL. RNA concentration was determined by measuring absorbance at 260 and 280 nm and purity calculated using ratios of absorbance at 260 and 280 nm (260/280). RNA integrity was checked by formaldehyde agarose gel electrophoresis. Briefly, each sample was added to one well of a 1.2% formaldehyde/agarose gel with ethidium bromide and electrophoresed at 5 V/cm. Bands were visualized with UV light and documented by capture with a Fuji LAS-3000 camera.

**RT-PCR:** Briefly, 2 µg of total RNA from each experimental sample was used in a reverse transcriptase (RT) reaction. Reaction conditions were: 1x RT buffer, 0.5 mM dNTP, 1 µM oligo-dT primer, 10 U/µL RNase inhibitor, and 4 U/µL RT enzyme in a total reaction volume of 20 µL. Each sample was incubated at 37° for 1 hour. Each completed RT reaction mix was added to a PCR master mix (Qiagen). The resulting PCR cocktail was aliquoted (25 µL) into PCR tubes containing appropriate primers (Super Array, Frederick, MD) for the gene of interest. PCR was performed with 30 cycles of the following program: 30 sec at 95° C, 30 sec at 55°, and 30 sec at 70° C. Following the completion of PCR, 10 µL of each sample was electrophoresed through a 2% agarose gel at 5 V/cm. Images were captured using a Fuji LAS-3000 camera. Densitometry of bands was analyzed using Multi-Gauge (Fujifilm USA, Burbank, CA). Data was normalized to expression of a housekeeping gene (GAPDH) and expressed as percent of control.
**Western Blotting:** HEL-30 keratinocytes, L929 fibroblasts, PAM-212 keratinocytes, or A.2R.1 fibroblasts were plated into cell culture plates and allowed to grow overnight. Cells were infected with HSV-1 as described above. The virus was removed and fresh DMEM containing 10% BCS added. At the indicated time points, medium was removed and cells were rinsed 3x with PBS. Cells were then lysed with Complete Lysis Buffer M (Roche Diagnostics, Indianapolis, IN) by following manufacturer’s suggestions. Equal amounts of lysate were combined with 6x Laemelli buffer and resolved by SDS-PAGE. Proteins were electro-blotted overnight onto PVDF. Membranes were blocked for one hour with 3% bovine serum albumin/TBS-Tween. Membranes were incubated with primary antibody to SOCS-1 (Millipore, Temecula, CA.) STAT-1, p-STAT-1 (Santa Cruz Biotech, Santa Cruz, CA) or β-Tubulin (Sigma ImmunoChemicals, St. Louis, MO). Membranes were rinsed 3x with TBS-Tween and then incubated with an appropriate secondary antibody (Santa Cruz Biotech). Membranes were striped using Restore Western Blot Stripping Buffer (Thermo Scientific, Rockford, IL) by following manufacturer’s suggestions. Proteins bands were resolved by chemiluminescence using Pierce ECL Reagent (Thermo Scientific). Images were captured as before using a Fuji LAS-3000 camera. Densitometry analysis was performed Multi-Gauge software as before.

**Cloning of SOCS-1 reporter construct:** A DNA fragment containing the human SOCS-1 promoter was amplified using genomic DNA purified from WISH cells. The forward and reverse primers used for amplification were 5’-TTTGCTAGCTCTTCCGCAGCCGGGTAGTG-3’ and 5’-TCCAAGCTTTACAGAAGGCGCCAGCCGGA-3’, respectively. The following
conditions were used for PCR: 94°C, 30 sec; 62°C, 30 sec; 68°C, 90 sec; for 30 cycles. The PCR fragment was purified and digested with Nhe I and Hind III and ligated with pGL3 basic reporter plasmid (Promega, Madison, WI) expressing firefly luciferase, digested with similar enzymes. The sequence of the reporter plasmid thus generated, which contained nucleotides -1577 to -3 of the promoter was confirmed by DNA sequencing.

**Luciferase Assay:** HEL-30 cells or L929 cells were plated into 12-well cell culture dishes and allowed to grow overnight. Cells were transfected with pGAS-Luc construct (Strategene, La Jolla, CA) or SOCS-1 promoter-linked firefly luciferase (pSOCS1-Luc) using GeneJammer transfection reagent (Stratagene) for the HEL-30 cells and Metafectene Pro (Biontex Laboratories) for the L929 cells. A constitutive reporter, pRL-SV40 (Stratagene) was co-transfected at a 1:20 ratio. Twenty-four hours after transfection, cells were either treated with 2000 U/mL murine IFN-γ or infected with HSV-1 at an MOI of 2.0 for 4 hours prior to treatment with IFN-γ. After treatment, cells were lysed with Passive Lysis Buffer. Lysates were then assayed for luciferase activity using Dual Luciferase Assay Kit (Promega). Relative Luciferase Units (RLUs) were calculated by dividing the observed luciferase activity of pGAS-Luc or pSOCS1-Luc by the observed activity of pRL-SV40. RLU levels were normalized to that of the untreated controls.

**SOCS-1 Transfection:** HEL-30 cells or L929 cells were plated into 12-well cell culture dishes and allowed to grow overnight. Cells were transfected with a construct containing the full-length murine SOCS-1 gene, pFLAG-SOCS-1 (a kind gift of Dr. Douglas Hilton, Walter and Eliza Hall Institute, Victoria, Australia). Briefly, L929 cells at ~90%
confluence were transfected with 4 µg/well of pFLAG-SOCS-1 using Metafectene Pro. Twenty-four hours later, cells were lysed and extracts used for Western blotting to confirm expression of the SOCS-1 protein. HEL-30 cells at ~50% confluence were transfected with the same amount of plasmid using Genejammer. HEL-30 cells were lysed and extracts used for Western blotting as above.

**siRNA Transfection:** HEL-30 cells were plated into 96-well cell culture plates and then incubated overnight. At 50% confluence, cells were transfected with a pool of SOCS-1 siRNA or control siRNA (Dharmacon RNAi Technologies, Boulder, CO) using Dharmafect 4 (Dharmacon). Total RNA was extracted 72 hours later using RNeasy as described above. RT-PCR was performed on samples as described above. For CPE assays, IFN-γ was added at the indicated concentrations 48 hours post-transfection and then plates were incubated for an additional 24 hours. Plates were then infected with HSV-1 at an MOI of 0.1 as above. Plates were incubated for 48 hours and rinsed and then stained as above.

**Statistical Analysis:** Statistical analysis was performed using Sigma Stat 11.0 (Jandell Corporation, San Rafael, CA). Values are expressed as ± SEM. The Mann-Whitney signed rank test was used to determine significance of treated groups versus untreated controls.
RESULTS:

IFN-γ induces an antiviral state against HSV-1 in fibroblasts but not keratinocytes

Keratinocytes are important for HSV-1 replication in the epidermis, which plays a role in infection of nervous tissue. The ability of IFN-γ to inhibit HSV-1 replication in HEL-30 keratinocytes relative to L929 fibroblasts was determined (Figure 5). As shown, IFN-γ at concentrations of 12.5 to 50 units/ml protected fibroblasts infected with HSV-1 at an MOI of 0.1, while HEL-30 keratinocytes were susceptible to HSV-1-mediated lysis at the same concentrations of IFN-γ. HSV-1 infection of keratinocyte (PAM-212) and fibroblast (A.2r.1) isolated from BALB/c mice showed similar responsiveness to IFN-γ (Figure 6). Comparable results from cell lines derived from mice of different genetic background indicate that resistance or sensitivity to the anti-viral actions of IFN-γ may be dependent on cell type. However, the lineage of each keratinocyte line may be different enough to explain the differences seen between HEL-30 and PAM-212 cells. These observations also suggest a possible basis for HSV-1 pathogenesis of epithelial cells as well as an approach to possible modulation of HSV-1 pathogenesis.

A small peptide mimetic of mouse IFN-γ consisting of the C-terminus of IFN-γ with an attached palmitate for plasma membrane penetration has been described (Thiam et al., 1999). The mimetic contains an essential alpha helix and polycationic nuclear localization sequence (Larkin et al., 2001). It binds to the cytoplasmic domain of the IFN-γ IFNGR1 receptor subunit and participates in activation of STAT1α (Subramaniam et al., 2000, 2001). The mimetic peptide, IFN-γ (95-132), inhibited HSV-1-induced CPE
at 30 μM in a manner similar to IFN-γ in L929 fibroblasts, while having little antiviral effect in HEL-30 keratinocytes (Figure 7).
Figure 5. Response to IFN-γ in L929 fibroblasts and HEL-30 keratinocytes infected with HSV-1. (A) L929 fibroblasts or (B) HEL-30 cells were treated with IFN-γ for 24 hours and infected with HSV-1 at MOI 0.1. Values are expressed as percentage of background corrected medium control. HSV-1 control wells were used as background and scored as zero. Error bars indicate standard error of the means. The experiments were carried out in triplicate and data are representative of duplicate wells in two independent experiments. There were statistically significant differences between IFN-γ treated cells and untreated cells (P < 0.001).
A. IFN-γ in HSV-1-infected L929 cells

B. IFN-γ in HSV-1-infected HEL-30 cells
Figure 6. Response to IFN-γ in PAM-212 keratinocytes and A.2r.1 fibroblasts infected with HSV-1. (A) PAM-212 keratinocytes or (B) A.2r.1 fibroblasts were treated with IFN-γ for 24 hours and infected with HSV-1 at MOI 0.1. Values are expressed as percentage of background corrected medium control. HSV-1 control wells were used as background and scored as zero. Error bars indicate standard error of the means. The experiments were carried out in triplicate and data are representative of duplicate wells in two independent experiments (n=4). There were statistically significant differences between IFN-γ treated cells and untreated cells (P < 0.001).
A. IFN-γ in HSV-1-infected PAM-212 cells

B. IFN-γ in HSV1-infected A.2r.1 cells
Figure 7. Response to IFN-γ peptide mimetics in L929 fibroblasts and HEL-30 keratinocytes infected with HSV-1. (A) L929 fibroblasts or (B) HEL-30 keratinocytes were treated with IFN-γ peptide mimetics for 24 hours and infected with HSV-1 at MOI 0.1. Values are expressed as percentage of background corrected medium control. HSV-1 control wells were used as background and scored as zero. Error bars indicate standard error of the means. The experiments were carried out in triplicate and data are representative of duplicate wells in two independent experiments (n=4). There were statistically significant differences between IFN-γ mimetic treated cells and untreated cells (P < 0.001).
A. IFN-γ mimetics in HSV-1-infected L929 cells

B. IFN-γ mimetics in HSV-1-infected HEL-30 cells
Both HSV-1 and IFN-γ strongly activate expression of SOCS-1 in keratinocytes

To determine a possible mechanism for the resistance of HEL-30 keratinocytes to induction of an antiviral state, activation of SOCS-1, a member of the SOCS family, was examined. There are eight known members of the SOCS family and SOCS-1 is particularly prominent in negative regulation of IFNs and cytokines that play an important role in immune functions (Alexander et al., 2004). In particular, overexpression of SOCS-1 can inhibit IFN-γ-induced gene activation in human keratinocytes (Federici et al., 2002).

End point RT-PCR analysis of SOCS-1 mRNA expression in HSV-1 infected fibroblasts and keratinocytes showed an increase of 5-fold for HEL-30 keratinocytes and a negligible increase for L929 fibroblasts (Figure 8). To study the effects of increasing viral load on induction of SOCS-1 message in HEL-30 cells, cells were infected with increasing MOIs for 2 hours. Based on end point RT-PCR SOCS-1 mRNA was increased at MOIs ranging from 1.0 to 5.0. Most noticeably, an MOI of 5.0 showed the greatest increase in SOCS-1 mRNA (Figure 9A). However, an MOI of 5.0 in HEL-30 cells results in complete cell death by 12 hours. Therefore, an MOI of 2.0 was used in subsequent experiments. A time-course of SOCS-1 mRNA expression in HSV-1-infected HEL-30 cells is shown in Figure 9B. Expression of SOCS-1 mRNA is elevated as early as 1 hpi and stays elevated past 6 hpi with maximal expression seen between 4 and 6 hpi. Densitometry analysis indicated that levels of SOCS-1 message double within 1 hpi (SOCS-1/GAPDH ratio: 0.14/0.31), with expression levels remaining elevated beyond 6 hpi (SOCS-1/GAPDH ratio: 0.14/0.39)
Western blots of HSV-1 infected cells showed increased SOCS-1 protein in keratinocytes (Figure 10A), with minimal expression in fibroblasts (Figure 10B). Densitometry analysis of SOCS-1 protein blots indicated that levels of SOCS-1 in HSV-1-infected HEL-30 cells doubled by two hpi (SOCS-1/β-Tubulin ratios: 0.39/0.78) and were nearly tripled by 6 hpi (SOCS-1/β-tubulin ratios: 0.39/1.09). These values are consistent with elevated levels of SOCS-1 mRNA observed early in infection (Figure 9B). HSV-1 infection of PAM-212 keratinocytes and A.2r.1 fibroblasts showed similar patterns of SOCS-1 induction (Figure 11). Densitometry analysis showed that SOCS-1 protein levels were nearly tripled at 1 hpi (SOCS-1/β-tubulin ratio: 0.015/0.044) and remained elevated through 4 hpi (SOCS-1/β-tubulin ratio: 0.015/0.039) in HSV-1-infected PAM-212 cells. Thus, keratinocytes showed a dramatic increase in SOCS-1 mRNA and protein after HSV-1 infection compared to both untreated cells and HSV-1 infected fibroblasts. This effect does not seem to be dependent on genetic background, since cell lines from two strains of mice showed comparable results. These observations are important in providing insight into the importance of keratinocytes in establishing HSV-1 infection and pathogenesis.
Figure 8. Induction of SOCS-1 mRNA in HSV-1-infected HEL-30 cells, but not in L929 cells. HEL-30 and L929 cells were infected with HSV-1 at MOI 1.0 for 12 hours. Total RNA was extracted and used as a template for end point RT-PCR using primers specific for SOCS-1 or GAPDH, the control. Ratios of SOCS-1 to GAPDH were determined by densitometry analysis using Multi-Gauge software. Data are presented as the ratio of SOCS-1 to GAPDH.
SOCS-1            GAPDH

Ctrl

HSV-1

HEL-30

Ctrl

HSV-1

HEL-30 + HSV-1

HSV-1

L929

HEL-30 ctrl

L929 ctrl

HEL-30 + HSV-1

L929 + HSV-1

SOCS-1/GAPDH Ratios

0.00

0.05

0.10

0.15

0.20

SOCS-1/GAPDH Ratios
Figure 9. Induction of SOCS-1 mRNA in HSV-1-infected HEL-30 cells is dependent on MOI and time. A). HEL-30 cells were infected with HSV-1 at the indicated MOI for 2 hours. B). HEL-30 cells were infected with HSV-1 at MOI 2.0 for the indicated times. Total RNA was extracted and used as a template for end point RT-PCR using primers specific for SOCS-1 or GAPDH, the control. Gels are representative of two independent experiments. Ratios of SOCS-1 to GAPDH were determined by densitometry analysis using Multi-Gauge software.
A. Dependence of SOCS-1 induction in HEL-30 on HSV-1 MOI

B. Dependence of SOCS-1 induction in HEL-30 on time post-infection
Figure 10. Induction of SOCS-1 protein in HSV-1-infected HEL-30 cells, but not in L929 cells. A) HEL-30 and B) L929 cells were infected with HSV-1 syn 17+ at MOI 2.0 for the indicated time points. Cellular proteins were extracted and electrophoresed on 10% SDS-PAGE. Proteins were electroblotted onto PVDF membranes. Membranes were blocked and probed with antibody for SOCS-1. Membranes were stripped and re-probed with an antibody to β-Tubulin to demonstrate equal protein loading. Blots are representative of two independent experiments. Ratios of SOCS-1 to β-Tubulin were determined by densitometry analysis using Multi-Gauge software.
A. SOCS-1 protein in HSV-1-infected HEL-30 cells

B. SOCS-1 protein in HSV-1-infected L929 cells
Figure 11. Induction of SOCS-1 protein in HSV-1-infected PAM-212 cells, but not in A.2r.1 cells. A) PAM-212 and B) A.2r.1 cells were infected with HSV-1 at MOI 2.0 for the indicated time points. Cellular proteins were extracted and electrophoresed on 10% SDS-PAGE. Proteins were electroblotted onto PVDF membranes. Membranes were blocked and probed with antibody for SOCS-1. Membranes were stripped and re-probed with an antibody to β-Tubulin to demonstrate equal protein loading. Ratios of SOCS-1 to β-Tubulin were determined by densitometry analysis using Multi-Gauge software. Blots are representative of two independent experiments.
A. SOCS-1 protein in HSV-1-infected PAM-212 cells

(-) 1 2 3 4 h.p.i

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<tr>
<th>SOCS-1</th>
<th>B-Tubulin</th>
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B. SOCS-1 protein in HSV-1-infected A.2r.1 cells

(-) 0 1 2 4 h.p.i

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<tr>
<th>SOCS-1</th>
<th>B-Tubulin</th>
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Hyperinduction of SOCS-1 by HSV-1 attenuates phosphorylation of STAT-1

SOCS-1 has been shown to bind to JAK-2, thereby hindering the autophosphorylation of JAK-2 and activation of downstream targets such as STAT-1 (Alexander et al., 2004). Previous studies have shown that overexpression of SOCS-1 strongly inhibits IFN-γ signaling in keratinocytes (Federici et al., 2002; Albanesi et al., 2002). Therefore, the effect of induction of SOCS-1 by HSV-1 on phospho-STAT-1 levels in IFN-γ-treated keratinocytes treated was studied. As shown in Figure 12, STAT-1 phosphorylation in HSV-1 infected HEL-30 cells is reduced to nearly background levels. Note that pSTAT-1 levels in HEL-30 cells may be reduced as compared to L929 cells based on the fact that HEL-30 blots required longer exposure times to visualize pSTAT-1 proteins. In contrast, levels of pSTAT-1 are not affected in HSV-1 infected L929 fibroblasts. This suggests that cells of the epidermis and dermis may have different mechanisms for combating infection by HSV-1. Interestingly, while these observations are in agreement with previously published reports showing a reduction in pSTAT-1 levels in HSV-1 infected cells of epithelial origins (Chee et al., 2004; Yokota et al., 2001), induction of SOCS-3 is the proposed mechanism for inhibition of IFN-γ signaling (Yokota et al., 2004).

HSV-1 and IFN-γ increase transcription at the SOCS-1 promoter in HEL-30 cells

Previous studies have used reporter assays to examine regulation of transcription from the SOCS-1 promoter (Hebenstreit et al., 2003). In order to investigate the mechanism by which HSV-1 is able to stimulate the induction of SOCS-1 shown in Figure 13, a luciferase reporter construct containing the full length SOCS-1 promoter was created. Transcription of the luciferase gene was increased in IFN-γ-treated
keratinocytes, and most noticeably increased in keratinocytes treated with both IFN-γ and HSV-1. Note that the increase in reporter activity in HSV-1-infected HEL-30 cells was larger than the increase in reporter activity with IFN-γ alone. Likewise, HSV-1 KOS stimulates transcription from the SOCS-1 promoter in a manner similar to HSV-1 syn 17+ (Figure 24). In contrast, HSV-1-infected L929 cells showed a decrease in reporter activity. These observations may explain the differences in expression of SOCS-1 in HSV-1-infected cells seen in Fig 10.

**Both HSV-1 and IFN-γ increase transcription of a reporter gene from GAS-containing promoters in HEL-30 cells**

Previous reports have indicated that HSV-1 infection inhibits IFN activity as measured from a reporter assay (Yokota et al., 2001). Given the large increase in SOCS-1 protein shown in Figure 10, the effect of HSV-1 infection of HEL-30 cells on IFN-γ signaling as measured by a GAS luciferase reporter assay was investigated. As shown in Figure 14, infection with HSV-1 prior to administration of IFN-γ did not inhibit luciferase activity in HEL-30 cells. Surprisingly, reporter activity was increased by infection with HSV-1 alone and further increased when cells were infected prior to IFN-γ treatment. In comparison to the HEL-30 cells, L929 fibroblasts showed no increase in reporter activity when infected with HSV-1. Moreover, L929 cells showed a larger increase in reporter activity when treated with IFN-γ alone than the HEL-30 cells (Figure 14).
Figure 12. Inhibition of STAT-1 activation in HEL-30 cells, but not L929 cells by infection with HSV-1. A) HEL-30 cells or B) L929 cells were serum-starved overnight and then infected with HSV-1 at MOI 2.0 for 4 hours and exposed to 1000 U/mL IFN-γ for 10 minutes. Whole cell extracts subjected to 10% SDS-PAGE. Proteins were blotted onto a PVDF membrane and probed with an antibody specific for pSTAT-1 (Tyr 701). Membranes were stripped and re-probed with an antibody to STAT-1α. Ratios of pSTAT-1 to total STAT-1 were determined by densitometry analysis using Multi-Gauge software. Blots are representative of two independent experiments.
A. Phospho-STAT-1 levels in HSV-1-infected HEL-30 cells

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<tr>
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<th>HSV-1</th>
<th>IFNγ</th>
<th>pSTAT1α</th>
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B. Phospho-STAT-1 levels in HSV-1-infected L929 cells

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<th>HSV-1</th>
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<th>pSTAT1α</th>
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Figure 13. HSV-1 increases transcription from the SOCS-1 promoter in HEL-30 cells but not in L929 cells. HEL-30 cells (black bars) and L929 cells (white bars) were transfected with a luciferase reporter construct containing the full-length SOCS-1 promoter. Cells were incubated for 24 hours and infected with HSV-1 at MOI 2.0 for 4 hours. Cells were then treated with 2000 U/mL IFN-γ for 2 hours. Cell lysates were collected and luciferase activity was measured in a single-tube luminometer. Values are representative of triplicate wells of two independent experiments (n=6). Error bars indicate standard error of the means. There were statistically significant differences between IFN-γ, HSV-1, and HSV-1 + IFN-γ treated cells when compared to the untreated cells (P < 0.001) as determined by Mann-Whitney signed rank test.
Figure 14. HSV-1 increases transcription from GAS elements in HEL-30 cells but not in L929 cells. HEL-30 cells (black bars) and L929 cells (white bars) were transfected with a plasmid containing luciferase and a 4X GAS-enhancer element. Cells were incubated for 24 hours and then mock-infected or infected with HSV-1 at an MOI of 2.0 for 4 hours. Cells were then treated with IFN-γ at 2000 U/mL for 2 hours. Cell lysates were collected and luciferase activity was measured in a single-tube luminometer. Error bars indicate standard error of the means. Values are representative of triplicate wells of two independent experiments (n=6).
Relative Luciferase Units

Medium IFN-γ HSV-1 HSV-1 + IFN-γ

A.

B.
Infection with HSV-1 inhibits expression of Type I IFNs in HEL-30 and L929 cells.

SOCS-1 can inhibit the actions of Type I IFNs (Fenner et al., 2006). Importantly, SOCS-1 dampens the anti-viral activity of these cytokines (Vlotides et al., 2004). Given the observation that infection with HSV-1 induces SOCS-1 in HEL-30 cells, the levels of Type I IFNs in HEL-30 cells and L929 cells were investigated. As shown in Figure 15, infection of HEL-30 cells with HSV-1 failed to induce expression of either IFN-α2 or IFN-β. This is contrary to previous results with mouse keratinocytes (Sprecher and Becker, 1992). The latter observations were in primary cells isolated from C57Bl/6 mice. There may be differences in expression patterns between primary cells and transformed cell lines or between different genetic backgrounds. Interestingly, infection of L929 cells with HSV-1 decreased expression of IFN-α2 and IFN-β nearly 4-fold (Figure 16). Consistent with previous observations, HEL-30 cells increased expression of TNF-α in response to HSV-1 infection (Figure 17).
Figure 15. HSV-1 inhibits expression of Type I IFNs in HSV-1-infected HEL-30 cells.  
A). Expression of IFN-α2 mRNA in HEL-30 cells.  B) Expression of IFN-β mRNA in HEL-30 cells. HEL-30 cells were infected at MOI 1.0 for 12 hours. Total RNA was extracted and used as a template for RT-PCR using primers specific for IFN-α/β or GAPDH, the control. Densitometry analysis of bands was performed using Multi-Gauge. Values are presented as the ratio of IFN-α/β to GAPDH.
A. IFN-α2 mRNA in HEL-30 cells

B. IFN-β mRNA in HEL-30 cells
Figure 16. HSV-1 inhibits expression of Type I IFNs in HSV-1-infected L929 cells.

A). Expression of IFN-α2 mRNA in L929 cells.  B) Expression of IFN-β mRNA in L929 cells. L929 cells were infected at MOI 1.0 for 12 hours. Total RNA was extracted and used as a template for RT-PCR using primers specific for IFN-α/β or GAPDH, the control. Densitometry analysis of bands was performed using Multi-Gauge. Values are presented as the ratio of IFN-α/β to GAPDH.
A. IFN-α2 mRNA in L929 cells

![Graph showing IFN-α2 mRNA levels in L929 cells untreated and with HSV-1](image)

B. IFN-β mRNA in L929 cells

![Graph showing IFN-β mRNA levels in L929 cells untreated and with HSV-1](image)
Figure 17. HSV-1 increases expression of TNF-α in HSV-1-infected HEL-30 cells.

HEL-30 cells were infected at MOI 1.0 for 12 hours. Total RNA was extracted and used as a template for RT-PCR using primers specific for TNF-α or GAPDH, the control. Densitometry analysis of bands was performed using Multi-Gauge. Values are presented as the ratio of TNF-α to GAPDH.
TNF-α in HEL-30 Cells

Ratio of TNF-a Gene to GAPDH Gene

Untreated vs +HSV-1

Bar graph showing the ratio of TNF-a gene to GAPDH gene. The untreated sample has a ratio of approximately 0.05, while the sample treated with +HSV-1 has a ratio of approximately 0.25.
A peptide antagonist of SOCS-1 partially restores anti-viral activity of IFN-γ in HSV-1 infected HEL-30 cells

A peptide antagonist of SOCS-1, known as pJAK2 has been previously characterized. Cells treated with pJAK2 and sub-threshold amounts of IFN-γ showed decreased CPE when infected with EMCV (Waiboci et al., 2007). Therefore, treatment of HEL-30 cells with pJAK2 and IFN-γ was studied. As shown in Figure 18, HEL-30 cells treated with pJAK2 and 100 U/mL IFN-γ exhibited reduced HSV-1-induced CPE as compared to IFN-γ alone. Specifically, cells treated with pJAK2 and IFN-γ showed ~76% cell survival while IFN-γ only cells showed ~58%. Interestingly, cells treated with pJAK2 alone showed a reduction in HSV-1-induced CPE as compared to untreated cells (Column 5 vs. Column 3). This suggests that pJAK2 is capable of inhibition of endogenous SOCS-1 in the HEL-30 cells, thereby permitting action of endogenous type I interferon. Previous studies showed that increased levels of SOCS-1 decreases IFN-α signaling, gene expression and anti-viral activity (Vlotides et al., 2004; Zimmerer et al., 2007). These results suggest that endogenous levels of SOCS-1 in the HEL-30 cells are responsible for the IFN resistance seen in Figure 5.

Knockdown of SOCS-1 partially restores antiviral activity of IFN-γ in HSV-1 infected HEL-30 cells

To corroborate the results shown with the antagonist peptides, siRNA targeted against SOCS-1 was used to knockdown expression in HEL-30 cells. As shown in Figure 19, HSV-1 induced CPE was inhibited by 1.5-2 fold in HEL-30 cells transfected with SOCS-1 siRNA prior to treatment with IFN-γ.
Figure 18. A peptide antagonist of SOCS-1 ameliorates HSV-1-induced CPE in HEL-30 cells. HEL-30 cells grown overnight in 24-well plates were treated with 100 U/mL IFN-γ alone, 35 μM pJAK2 alone or 35 μM pJAK2 with 100 U/mL IFN-γ. Following treatment for 24 hours, cells were mock-infected or infected with HSV-1 at an MOI of 0.1. Values are expressed as percentage of background corrected medium control. HSV-1 control wells were used as background and scored as zero. Error bars indicate standard error of the means. Values are expressed as percent cell survival relative to mock-infected controls. Values are representative of duplicate wells of two independent experiments (n=4).
<table>
<thead>
<tr>
<th>Treatment</th>
<th>HSV-1</th>
<th>IFN-γ</th>
<th>pJAK2, μM</th>
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% Cell Survival:

- 100
- 75
- 50
- 25
- 0
Figure 19. HSV-1-induced cytopathic effect is ameliorated by treatment with SOCS-siRNA. HEL-30 cells were transfected with control or SOCS-1 siRNA, incubated for 48 hours, then treated with indicated amounts of IFN-γ for 6 hours, and subsequently infected with 100 pfu HSV-1 (syn17+). Values are expressed as percent cell survival relative to mock-infected controls. Error bars indicate standard error of the means.
A SOCS-1 peptide mimetic, T-kip, inhibits the antiviral activity of IFN-γ in L929 fibroblasts

T-Kip, a peptide mimetic of SOCS-1, inhibits anti-viral activities of IFN-γ against several other viruses (Flowers et al., 2004). It was hypothesized that T-kip could act in a similar manner in L929 fibroblasts. As shown in Figure 20, L929 cells treated with T-kip demonstrated reduced survival when challenged with HSV-1 at an MOI of 0.1. Specifically, T-kip at concentrations from 5-20 µM reduced survival by at least 50%.

Overexpression of SOCS-1 in L929 cells inhibits the antiviral activity of IFN-γ

To confirm the role played by SOCS-1 in inhibition of the anti-viral effects of IFN-γ, L929 cells were transfected with a construct containing the full-length murine SOCS-1 gene. As shown in Figure 21, overexpression of SOCS-1 in L929 cells blocked the anti-viral actions of IFN-γ. Note that as little as 1µg of DNA was able to reduce survival of cells treated with IFN-γ prior to infection with HSV-1. Cells transfected with 2 µg of pFLAG-SOCS-1 showed 5-times less survival than cells treated with 1 µg of pFLAG-SOCS-1.
Figure 20. HSV-1-induced CPE is increased in L929 cells treated with a SOCS-1 peptide mimetic. L929 fibroblasts were treated with IFN-γ and T-kip at the indicated concentrations for 24 hours, after which HSV-1 (syn 17+) was added at an MOI of 0.1. Values are expressed as percentage of background corrected medium control. HSV-1 control wells were used as background and scored as zero. Error bars indicate standard error of the means. Values are representative of duplicate wells of two independent experiments (n=4).
Figure 21. Overexpression of SOCS-1 inhibits the anti-viral activity of IFN-γ in HSV-1-infected L929 cells. 

A) L929 fibroblasts were transfected with the indicated amounts of pFLAG-SOCS-1 plasmid. Cells were incubated for 24 hours and treated with 100 U/mL IFN-γ for 24 hours. Cells were infected with HSV-1 (syn 17+) at an MOI of 0.1. Data is expressed as percentage of background corrected medium control. HSV-1 control wells were used as background and scored as zero. Error bars indicate standard error of the means. Values are expressed as percent cell survival relative to mock-infected controls. Values are representative of duplicate wells of two independent experiments (n=4).

B) Transfection with cDNA expressing SOCS-1. L929 cells were transfected with SOCS-1 expression plasmid for one day. Cell extracts were then electrophoresed and probed with an antibody to SOCS-1 followed by stripping and probing with ß-tubulin antibody as a control.
A. Increased cytopathic effect of HSV-1 in L929 cells

B. Overexpression of SOCS-1 in L929 cells
Disruption of the HSV-1 IE Gene ICP0 retards the induction of SOCS-1 in HEL-30 cells

ICP0 is an IE virulence protein that increases expression of HSV-1 genes in infected cells (Chen and Silverstein, 1992; Cai and Shaffer, 1992). One way that it functions is by blockage of histone deacetylation and/or increase in histone acetylation to facilitate HSV-1 gene expression (Lomonte et al., 2004). It also causes degradation of host proteins that are involved in silencing HSV gene expression, such as the promyelocytic leukemia protein (Everett et al., 2006). To determine if ICP0 might play a role in HSV-1 refractiveness to IFN-γ in HEL-30 keratinocytes, cells were infected with HSV-1 syn17+ that is ICP0-null (HSV-1 dl1403) (Stow and Stow, 1986). HSV-1 dl1403 was similarly lytic for HEL-30 cells as wild type virus, but unlike the wild type virus was inhibited by IFN-γ (Figure 22). Refractiveness to IFN-γ was restored when the cells were treated with the SOCS-1 mimetic, Tkip, at the time of IFN-γ treatment where 100 U/ml of IFN-γ activity was significantly blocked by as little as 2.5 µM of SOCS-1 mimetic (Figure 23). To assess as to how all this might be related to ICP0, SOCS-1, and IFN-γ in HEL-30 keratinocytes, cells were transfected with a luciferase reporter containing the SOCS-1 promoter (nucleotides -1577 to -3). Transfected cells were subsequently infected with wild type HSV-1 and HSV-1 dl1403 and compared for reporter gene activation. As shown in Figure 24, wild type HSV-1 was more than 2-fold more effective than dl1403 in activation of the reporter gene at comparable levels of infectivity. An ICP0 mutant of the KOS strain showed essentially similar results (Figure 24). These observations suggest that ICP0 has a direct or indirect effect on SOCS-1 gene activation in keratinocytes.
Figure 22. HEL-30 cells infected with an ICP0 mutant of HSV-1 are responsive to IFN-γ treatment. HEL-30 cells were cultured with indicated amounts of IFN-γ. Following treatment for 24 hours, cells were mock-infected or infected with HSV-1 dl1403 at MOI of 0.1. Values are expressed as percentage of background corrected medium control. HSV-1 control wells were used as background and scored as zero. Error bars indicate standard error of the means. Values are representative of duplicate wells of two independent experiments (n=4).
Figure 23. Tkip abrogates IFN-γ sensitivity HEL-30 infected with HSV-1 dl1403. HEL-30 cells were treated with 100 U/ml IFN γ alone and with different concentrations of Tkip. Following treatment for 24 hours, cells were mock-infected or infected with HSV-1 dl1403 mutant at MOI of 0.1. Values are expressed as percentage of background corrected medium control. HSV-1 control wells were used as background and scored as zero. Error bars indicate standard error of the means. Values are representative of duplicate wells of two independent experiments (n=4). There were statistically significant differences between IFN-γ, Tkip, IFN-γ + HSV-1 and Tkip + HSV-1 when compared to the untreated cells (P < 0.001) as determined by Mann-Whitney signed rank test.
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Figure 24. Reduction in SOCS-1 gene activation by HSV-1 ICP0 mutant in HEL-30 keratinocytes. HEL-30 cells were transfected with a luciferase reporter construct containing the full-length SOCS-1 promoter. Cells were incubated for 24 hours and then mock-infected or infected with (A) HSV-1 syn 17\(^+\) or HSV-1 \textit{dl1403} at MOI of 2 for 4 hours. (B) Cells were infected with HSV-1 KOS or its ICP0\(^-\) mutant. Cell lysates were collected and luciferase activity was measured in a single-tube luminometer. Values given are expressed as luciferase units measured from the SOCS-1 reporter divided by luciferase units measured from a co-transfected constitutive reporter and subsequently normalized to medium controls. Values are representative of triplicate wells of two independent experiments.
A. Reduction of reporter activity at the SOCS-1 promoter by HSV-1 dl1403

B. Induction of reporter activity from the SOCS-1 promoter by HSV-1 KOS
DISCUSSION

Epithelial cells such as keratinocytes are the major site of HSV-1 replication in active primary or recurring herpes infection (Cunningham et al., 2006). After virus replication and subsequent lysis of keratinocytes, HSV-1 then accesses nerve terminals in the epidermis and travels to the neuronal soma via retrograde transport. Once in the neuron, the virus establishes a latent state, which is maintained by CD8+ T-cells and IFN-γ (Hendricks et al., 1992).

In studying the effects of mouse IFN-γ and an IFN-γ peptide mimic on HSV-1-infected cell lines, a difference was seen in the antiviral effects of these substances on fibroblast (L929) and keratinocyte (HEL 30) cell lines derived from C3H mice. L929 fibroblasts were protected from HSV-1-mediated cytopathic effect (CPE) while the HEL-30 keratinocytes were not (Figures 5). Significantly, L929 cells were protected from CPE at levels as low as 12.5 U/mL of IFN-γ while HEL-30 cells were not protected at any concentration tested in this experiment. The antiviral effects of IFN-γ following HSV-1 infection of another murine keratinocyte cell line derived from BALB/c mice (PAM-212), was similar to that seen in HEL-30 cells (Figure 6). Similar to L929 cells, a fibroblast cell line from BALB/C mice (A.2r.1) was protected from viral-mediated CPE by pre-treatment with IFN-γ (Figure 10). Similarly, a peptide mimic of IFN-γ was able to protect L929 cells from HSV-1-induced CPE, but not the HEL-30 cells (Figure 7).
The peptide mimetic of IFN-γ contains an essential nuclear localization signal (Larkin et al., 2001) and protects mice against lethal infection with vaccinia virus or encephalomyocarditis virus in mice (Mujtaba et al., 2006; Ahmed et al., 2007). Oral administration of the mimetics also provided complete protection against vaccinia lethality (Ahmed et al., 2007). *In vitro* results suggest that treatment with IFN-γ peptide mimetics can increase survival of HSV-1-infected cells (Figure 7). These peptides may provide protection against lethal challenge with HSV-1 in mice. Mice treated with IFN-γ peptide mimetics also show increased survival and enhanced immune responses when re-challenged with vaccinia virus (Ahmed et al., 2007). Since HSV-1 emerges from a latent state and effectively re-challenges the host (Sheridan et al., 2007), the results using vaccinia may have implications in HSV-1 infections. The observations of a differential response to HSV-1 and IFN-γ in C3H keratinocytes and fibroblasts led to investigation of the role of SOCS-1, an important negative regulator of IFN-γ signaling (Alexander et al., 1999).

In this study, the results of an initial screen of mRNA levels in the two cell types showed a large increase in SOCS-1 mRNA in response to HSV-1 infection and IFNγ treatment in the HEL-30 cells, but not in the L929 cells (Figure 8). Specifically, mRNA levels of SOCS- in HSV-1 infected keratinocytes were 5-fold higher than endogenous levels. In contrast, negligible levels of SOCS-1 mRNA were detected in both normal and HSV-1-infected L929 cells. Additionally, use of higher viral MOIs increased induction of SOCS-1 during the first 6 hours of infection (Figure 9). Expression of SOCS-1 protein in HSV-1-infected HEL-30 and L929 cells correlated with mRNA levels (Figure 10). These data showing induction of SOCS-1 in HSV-1-infected keratinocytes are further
supported by observations showing degradation of JAK2 in HSV-1-infected HeLa cells (Chee and Roizmann, 2004) since SOCS-1 is capable of targeting JAK2 for proteosomal degradation via its E3-ubiquitin ligase domain (Kile et al., 2004). These findings are important in providing insight into the role of keratinocytes in establishing HSV-1 infection since IFN-γ is a potent activator of keratinocytes as well as an inducer of SOCS-1 (Albanesi et al., 1998).

HEL-30 keratinocytes (Figure 10) and PAM-212 (Figure 11) keratinocytes express higher levels of endogenous SOCS-1 than do their fibroblast counterparts. Why might these keratinocyte cells express SOCS-1 constitutively? Keratinocytes differ in gene expression programs depending on location. Expression of keratin proteins and transcription factors shifts as cells differentiate (Bowden et al., 1987; Eckhert et al., 2004). More recent evidence suggests that expression of genes related to interferon signaling changes as cells differentiate. Specifically, STAT-1, OAS and MX1 were more highly expressed in keratinocytes representative of the stratum granulosum (Perera et al., 2006). This suggests that keratinocytes become more active in the immune response as they differentiate. Since HEL-30 and PAM-212 cells are still replicative and representative of cells of the stratum basale, it could be beneficial to dampen the more deleterious effects of inflammation. Furthermore, KCs are strongly activated by IFN-γ (Mikloska and Cunningham, 1998; Albanesi et al., 1998). Hence, it may be advantageous to regulate the response to IFN-γ by only reacting to higher levels of IFN-γ. Similarly, differential expression of SOCS-1 may be reflective of differential regulation of signaling pathways.
Overexpression of SOCS-1 in keratinocytes inhibits the biological effects of IFN-\(\gamma\) (Federici et al., 2002). Importantly, IFN-\(\gamma\) can upregulate MHC class II molecules, induce expression of ICAM-1, and reverse viral-mediated downregulation of MHC class I molecules (Mikloska et al., 1996; Mikloska and Cummingham, 1998). IFN-\(\gamma\) is especially important in maintaining HSV-1 in a latent state in neurons (Khanna et al., 2004). Consequently, disruption of IFN-\(\gamma\) signaling may provide an important survival advantage to the replicating virus.

Inhibition of INF-\(\gamma\) by HSV-1 may impact the progression of other infections. Human immunodeficiency virus 1 (HIV-) upregulates SOCS-1 expression in dendritic cells (Yadav et al., 2008). This finding has importance in public health given that HSV-1-infected individuals are more susceptible to infection with HIV-1 (Van de Perre et al., 2008). HSV-1-infected epithelial cells may create a more permissive environment for HIV-1 infection by dampening local inflammation. Finally, HSV-1 is not the only family member to alter expression of SOCS-1. Epstein-Barr virus, a member of the \(\gamma\)-Herpesvirus family, upregulates SOCS-1 in nasopharyngeal epithelial cells (Lo et al., 2006).

Moreover, a number of micro-organisms induce expression of SOCS-1. Flagellin, a bacterial protein present in motile bacteria, induced expression of SOCS-1 in CD4\(^+\) T cells. Expression of SOCS-1 in these cells was correlated with inhibition of T cell activation. SiRNA-mediated knockdown of SOCS-1 reversed the inhibition (Okugawa et al., 2006). Similarly, two intracellular pathogens, *Mycobacterium avium* and *Toxoplasma gondii* induce SOCS-1 expression in macrophages. Increased expression of SOCS-1 creates insensitivity to IFN-\(\gamma\) in infected macrophages, permitting increased microbial
survival (Vazquez et al., 2006; Zimmerman et al., 2006). Accordingly, inhibition of IFN-γ signaling by induction of SOCS-1 may represent a conserved pathway for immune evasion among multiple classes of micro-organisms.

SOCS-1 inhibits IFN-γ signaling via binding to JAK2 and thereby inhibiting the phosphorylation of downstream targets such as STAT-1 (Alexander et al., 2004). Western blot results show diminished levels of pSTAT-1 in HEL-30 cells infected with HSV-1 prior to treatment with IFN-γ (Figure 12). This observation is in agreement with previously published reports (Yokota et al., 2001; Chee et al., 2004). Induction of SOCS-3 was postulated as a mechanism for inhibition of IFN-γ-stimulated activation of STAT-1. Yet, overexpression of SOCS-3 was not shown to inhibit IFN-γ signaling (Yokota et al., 2004). L929 fibroblasts express SOCS-3 mRNA constitutively (Appendix B) but do not show defects in IFN-γ activation of STAT-1. Additionally, HSV-1 infected L929 cells showed no reduction of pSTAT-1 (Figure 12). In contrast, overexpression of SOCS-1 obstructs the antiviral effects of IFN-γ in L929 cells (Figure 21). Similarly, a peptide mimetic of SOCS-1 inhibited the effects of IFN-γ in L929 cells (Figure 20). These data argue that SOCS-1 is the primary regulator of IFN-γ signaling.

It is noteworthy that previous reports demonstrating inhibition of IFN signaling were performed in cell lines of epithelial origin (Yokota et al., 2001; Chee et al., 2004). These observations suggest that the ability of HSV-1 to inhibit IFN signaling may be dependent on cell type.

The ability of HSV-1 to induce large amounts of SOCS-1 in the HEL-30 cells may be mediated at the transcriptional level. Consequently, whether or not HSV-1 could stimulate transcription from the SOCS-1 promoter was tested. As shown in Figure 13,
HSV-1 alone was able to stimulate a large increase in reporter activity in HEL-30 cells. No increase was noted in HSV-1-infected fibroblasts, again reflecting the differential responses to HSV-1 in these two cell lines.

A reporter assay from HEL-30 cells transfected with a construct containing a 4x GAS enhancer element showed that HSV-1 was able to stimulate a large increase in reporter activity, dwarfing that seen in cells treated with IFN-γ alone (Figure 14). Unexpectedly, data from cells infected with HSV-1 prior to treatment with IFN-γ showed no decrease in reporter activity. These data are in direct contrast to a previously published report in human FL cells infected with HSV-1 (VR3) (Yokota et al., 2001). FL cells, an amniotic cell line, were shown to contain HeLa marker chromosomes (ATCC.org). These disparate observations may indicate host-specific, cell-specific or virus strain-specific differences. Specifically, HEL-30 cells are keratinocytes derived from epidermis while HeLa cells were originally derived from cervical epithelium (Scherer et al., 1953).

The resistance of wild type HSV-1 to IFN-γ in HEL-30 cells is dependent on ICP0, since the ICP0 mutant was inhibited by IFN-γ (Figure 22), similar to the inhibition of wild type virus in fibroblasts. ICP0 is an HSV-1 IE protein that negates the silencing of virus gene by infected cells (Chen and Silverstein, 1992; Cai and Shaffer, 1992). The mechanism of this negation is thought to primarily involve blockage of histone deacetylation and/or increase in histone acetylation (Lomonte et al., 2004). ICP0 also causes degradation of host proteins such as the promyelocytic leukemia protein (Everett et al., 2006). The data shown here indicate that induction of SOCS-1 is the mechanism of HSV-1 resistance to IFN-γ in the keratinocytes. In this regard the HSV-1 ICP0 mutant
was less effective at activation of a luciferase reporter gene driven by the SOCS-1 promoter than the wild type virus (Figure 24). The question arises as to whether the activation of a host gene such as SOCS-1 by ICP0 occurs indirectly as per the mechanism above or by transcription/cotranscription mechanisms. ICP0 is not known to bind to DNA (Lomonte et al., 2004), but this has not been extensively examined with respect to host genes.

The influence of SOCS-1 on responsiveness to IFN-\(\gamma\) in these two C3H cell lines was further examined using a peptide antagonist of SOCS-1 in the keratinocytes, and a peptide mimetic of SOCS-1 in the fibroblasts. Previous studies showed that a peptide antagonist of SOCS-1, pJAK2, binds the SOCS-1 kinase inhibitory region in a dose-dependent manner (Waiboci et al., 2007). Treatment of HEL-30 cells with pJAK2 and IFN-\(\gamma\) increased cell viability following HSV-1 infection (Figure 18). This demonstrates that pJAK2 inhibits the actions of endogenous SOCS-1 in the HEL-30 cells. These observations were supported by the use of SOCS-1 siRNA in HEL-30 cells (Figure 22).

T-kip binds the JAK2 autophosphorylation loop in a dose-dependent manner (Flowers et al., 2004). Treatment of L929 cells with T-kip and IFN-\(\gamma\) reduced cell viability following infection with HSV-1 (Figure 20). Overexpression of SOCS-1 in the IFN-\(\gamma\)-treated L929 cells showed similar reductions in cell viability following HSV-1 infection (Figure 21). Taken together these data suggest that SOCS-1 plays a central role in the regulation of the anti-viral effects of IFN-\(\gamma\).

Since SOCS-1 also affects activity of type I IFNs (Fenner et al., 2006), inhibition of signaling by endogenously expressed type I IFN may play a role in the differential responses observed in the two cell lines. Specifically, IFN-\(\gamma\) signaling is dependent on
interactions of IFNGRs with Type I receptors in caveolae (Takaoka et al., 2000). The implications of this observation for the data presented here are three-fold: (i) Increased expression of SOCS-1 in HSV-1-infected cells may dampen the actions of endogenously expressed Type I IFNs thereby preventing interactions with IFNGRs; (ii) Blockade of SOCS-1 function by pJAK2 may relieve the inhibition of Type I IFNs in HSV-1-infected cells; (iii) Once the inhibition of Type I IFNs is relieved, exogenously applied IFN-γ may synergize with Type I IFNs to inhibit replication of HSV-1. Indeed, one may speculate that relief of Type I inhibition by pJAK2 is the mechanism underlying the reduction of CPE in HEL-30 cells treated with pJAK2 alone (Figure21 Column 5). However this effect is not as robust as the combination of pJAK2 and IFN-γ, as the effect is quickly diluted away (Figure 18, Column 7). Furthermore, preliminary results with HSV-1-infected L929 cells pretreated with neutralizing antibody to IFN-β indicate that inhibition of IFN-β decreases IFN-γ mediated cell survival (data not shown).

Strategies for treatment of recurrent HSV-1 disease have mainly focused on development of vaccines, an approach with mixed results (Sheridan et al., 2007). Conversely, a number of small molecule inhibitors have been investigated (reviewed by Weber, 2002). For instance, the best known inhibitors of HSV-1 are members of the acyclovir family, nucleoside analogs that inhibit viral DNA replication (reviewed by DeClercq and Neyts, 2008). One study indicated that quinine sulfate, an anti-malarial drug, suppresses HSV-1 replication in a human keratinocytes line (Baroni et al., 2007). These drugs often have undesirable side effects. Also, mutant viruses can escape the effects of the acyclovir family (DeClercq and Neyts, 2008).
Control of latency may be a more practical approach. IFN-γ is critical for maintaining HSV-1 latency (Liu et al., 2001; Decman et al., 2005). In particular, IFN-γ and TNF-α act synergistically to block HSV-1 replication (Feduchi et al., 1989, 1991). TNF-α has also been implicated in control of primary HSV-1 infection (Minigawa et al., 2004). This is especially important given recent reports showing that SOCS-1 can block TNF-α signaling (Morita et al., 2000; He et al., 2006). Consequently, use of IFN-γ peptide mimetics may prevent recrudescence in HSV-1-infected animals.

Suppression of SOCS-1 represents an innovative approach to treatment of viral infections. A peptide inhibitor of SOCS-1, pJAK2, can increase survival of EMCV-infected cells treated with sub-optimal doses of IFNγ (Waiboci et al., 2007). Likewise, inhibition of SOCS-1 increased survival of enterovirus-infected cardiomyocytes. This effect was mediated by overexpression with a dominant-negative construct of SOCS-1. Specifically, the dominant negative protein contains a point mutation in the kinase inhibitory region of SOCS-1 (Yasukawa et al., 2003). Similarly, suppression of SOCS-3 with anti-sense RNA was effective in limiting replication of HSV-1 in a human cell culture model (Yokota et al., 2005). These findings mirror the efficacy of treatment with pJAK2 in a cell line that is refractory to treatment with IFN-γ (Figure 18) and suggest that inhibition of SOCS-1 function is an effective method of increasing the efficacy of the antiviral effects of IFNγ.

Use of pJAK2 may act to enhance TNF-α-induced inflammation since SOCS-1 can negatively regulate TNF-α signaling (He, et al., 2006; Kimura et al., 2004). TNF-α can protect mice from lethal HSV-1 infection (Rossol-Voth et al., 1991). More importantly, TNF-α synergizes with IFN-γ to inhibit HSV-1 (Feduchi et al., 1989).
Further, pJAK2 may be able to increase IFN-γ-dependent expression of an important complement protein, C1ra (Byun et al., 2007). This may reverse HSV-1-mediated inhibition of the complement system (Kostavasili et al., 1997).

Certain evidence suggests that SOCS-1 can influence TLR signaling (Mansell et al., 2006). It has been observed that dsRNA stimulates production of anti-viral genes in epithelial cells through interactions with TLR3 (Kariko et al., 2004, Tohyama et al., 2005). Chick embryo cells showed increased resistance to viral infection after treatment with IFN-α and dsRNA (Marcus and Sekellick, 2001). Thus, SOCS-1 may impact a TLR3-mediated anti-viral pathway. In fact, treatment with pJAK2 increased the anti-viral effects of IFN-γ in murine macrophages (Appendix A). Further, a SOCS-1 mimetic reduced macrophage activity in response to lipopolysaccharide. This is most likely mediated via binding of T-kip to a TLR adaptor protein, MAL (HM Johnson, personal communication). Therefore, suppression of SOCS-1 function may act to augment several cytokine pathways involved in inflammation and viral resistance.

Combinatorial therapy with IFN-γ and immune modulators represents a mode of treatment for recurrent HSV-1 infection. For instance, treatment of HSV-1 lesions with caffeine potentiated the effects of topical IFN-γ in human subjects (Vonka et al., 1995). It is tempting to speculate that combination therapy with IFN-γ mimetics and pJAK2 may reduce virus-induced mortality and morbidity in HSV-1-infected animals.
FUTURE STUDIES

The cell culture studies presented here were conducted with murine cells and may not be reflective of responses in human cells. Replication of the results using a human keratinocyte line would corroborate these studies. Furthermore, use of primary human keratinocytes and fibroblasts would substantiate the conclusion that HSV-1 induces SOCS-1 in keratinocytes.

The use of pJAK2 to potentiate the antiviral actions of IFN-γ has been described (Waiboci et al., 2007). Treatment of IFN-γ-responsive cell lines with sub-optimal amounts of IFN-γ and pJAK2 could potentially inhibit HSV-1-induced CPE. In fact, preliminary data using L929 cells show that pJAK2 can potentiate amounts of IFN-γ as low as 2 U/mL (data not shown).

Since IFN-γ signaling is dependent upon interactions of Type I IFNs, treatment of cells with pJAK2 prior to infection could conceivably increase levels of Type I expression in HSV-1-infected cells. This could be an important result since HSV-1 is known to inhibit expression of Type I IFNs (Figure 18,19). Further, pJAK2 may increase expression of TNF-α. A simple assay such as ELISA could quickly determine if this hypothesis is true.

SOCS-1 can impact TLR signaling. Keratinocytes and fibroblasts express TLR2 and TLR3. Treatment of cells with pJAK2 could reduce SOCS-1 inhibition of TLR signaling. Again, use of an ELISA to detect levels of IFN-α/β would be a first step in testing this hypothesis.
APPENDIX A

pJAK2(1001-1013) synergizes with IFN-γ to protect RAW264.7 murine macrophages against HSV-1. Murine macrophage cell line RAW264.7 was treated with IFN-γ, pJAK2(1001-1013), IFN-γ and different concentrations of pJAK2(1001-1013), or IFN-γ and different concentrations of an alanine substituted mutant pJAK2(1001-1013)2A, followed by infection with HSV-1 at an moi of 0.1. Data is expressed as percentage of background corrected medium control. HSV-1 control wells were used as background and scored as zero. Error bars indicate standard error of the means. Values are representative of duplicate wells of two independent experiments. There were statistically significant differences between different concentrations of pJAK2 peptide and IFN-γ when compared to untreated cells (P < 0.001) as determined by Mann-Whitney signed rank test.
Untreated
None
pJAK2, 25 µM
IFNγ, 100 U/ml
pJAK2, 12.5 µM
pJAK2, 6.25 µM
pJAK2, 3.1 µM
JAK2A, 25 µM
JAK2A, 12.5 µM

% Cell Survival

+ IFNγ, 100 U/ml
+ HSV-1
APPENDIX B

Endogenous expression pattern of select genes in L929 cells. Agarose gel showing basal levels of mRNA for select genes in L929 cells. Each band represents an RT-PCR product amplified from cDNA using primers specific to each gene.

Lane 1: Marker
Lane 2: Blank
Lane 3: IFN-α2
Lane 4: SOCS-1
Lane 5: IFN-β
Lane 6: SOCS-3
Lane 7: IL-1α
Lane 8: MyD88
Lane 9: IL-6
Lane 10: IFGR1
Lane 11: IL-15
Lane 12: H2-D
Lane 13: TNF-α
Lane 14: GAPDH
APPENDIX C

Endogenous expression pattern of select genes in HEL-30 cells. Agarose gel showing basal levels of mRNA for select genes in HEL-30 cells. Each band represents an RT-PCR product amplified from cDNA using primers specific to each gene.

Lane 1: Marker
Lane 2: Blank
Lane 3: IFN-α2
Lane 4: SOCS-1
Lane 5: IFN-β
Lane 6: SOCS-3
Lane 7: IL-1α
Lane 8: MyD88
Lane 9: IL-6
Lane 10: IFGR1
Lane 11: IL-15
Lane 12: H2-D
Lane 13: TNF-α
Lane 14: GAPDH
APPENDIX D

Schematic representation of the interaction between HSV-1 and SOCS-1. HSV-1 induces expression of SOCS-1 through an unknown mechanism that probably involves expression of viral immediate early genes (IE) such as ICP0 and ICP4. Increased levels of SOCS-1 subsequently inhibit activation of JAK-STAT signaling. Treatment with pJAK2 is able to block the actions of SOCS-1.
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