Differential Expression of IFN-Gamma Stimulated Antiviral Genes in Human Herpes Simplex Virus Infection

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Abstract

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Herpes simplex virus type 2 (HSV-2) causes highly prevalent and recurrent ulcerative disease that affects millions of individuals worldwide. Clinical outcomes vary widely, ranging from asymptomatic, subclinical illness to severe, painful ulcerations. HSV-2 prevalence is further associated with an elevated risk of human immunodeficiency virus (HIV) acquisition, due to the recruitment of HIV-susceptible CD4+ T cells to sites of mucocutaneous infection. As HSV can establish latency in sensory ganglia and reactivate throughout an individual's lifetime, a host's innate and adaptive immune system must continuously surveil and contain viral activity. Viral containment is primarily attributed to HSV-specific CD8+ T cells that persist at sites of prior infection, alongside the network of chemokines and cytokines that coordinate local antiviral immune responses. Within the herpes lesion microenvironment, interferon gamma (IFN-γ) plays a critical role in viral control by inducing the expression of interferon-stimulated genes (ISGs) in neighboring cells, most notably keratinocytes and fibroblasts, which produce antiviral proteins, microbicidal molecules, phagocytic receptors, and additional immune modulators that help establish an antiviral state. In this study, we investigated donor-dependent IFN–γ–induced gene expression in primary structural cells isolated from individuals with asymptomatic or severe HSV-2 disease. By assessing the transcriptional response of specific ISGs in keratinocytes and fibroblasts exposed to IFN-γ, we identified distinct patterns of gene expression based on both donor phenotype and cell type. Our findings suggest that variability in the magnitude and regulation of IFN-γ-responsive genes may underlie the heterogeneous clinical manifestations of HSV-2 infection. Understanding the mechanisms that govern effective immune control can help us identify determinants of disease severity and inform the development of targeted therapeutic strategies aimed at long-term containment and eradication of HSV-2.

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Chapter 1	**Introduction** Chapter 1 provides a review of herpes simplex virus pathology, with a focus on the host immune response, including the role of cytokines and cytokine-mediated antiviral signaling.
Chapter 2	**Methods** Chapter 2 outlines the primary methods used in this study, including transcriptional profiling of IFN-γ induced gene expression, cell culture and infection with HSV.
Chapter 3	**Data and Results** Chapter 3 provides a summary of key findings and statistical analyses.
Chapter 4	**Discussion** Chapter 4 extends the discussion of key findings, including implications, limitations and future directions of the study.

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Chapter 1. Introduction

1.1 HSV Virology and Epidemiology

The herpesviruses are a class of enveloped double-stranded DNA viruses that establish lifelong latent infection with episodic recurrences in susceptible hosts [1]. A total of eight herpesviruses are known to infect humans, including herpes simplex virus type 1 (HSV-1) and herpes simplex virus type 2 (HSV-2). HSV-1 and HSV-2 belong to the subfamily *Alphaherpesvirinae* and cause highly prevalent infection in the human population [1,2,9].

An estimated 3.7 billion individuals under the age of 50 harbor HSV-1 globally [7]. Most individuals acquire HSV-1 early in life through viral contact with the oral and labial mucosa, with the disease manifesting as cold sores or fever blisters around the mouth during reactivation [1]. HSV-2 is one of the most common sexually transmitted infections worldwide and infects over 500 million people globally [7,13]. Unlike HSV-1, HSV-2 infections tend to occur later, typically through sexual contact with an individual shedding virus [1,2]. While the rate of HSV-2 coital acquisition and serological prevalence of HSV-2 is higher in women than in men, both groups are susceptible to a two-to threefold increased risk of HIV infection after acquiring HSV-2 [13]. Likewise, HSV-1 and HSV-2 establish lifelong latency within the peripheral sensory neurons, where the virus remains until episodic reactivation triggers a productive infection. While most individuals harbor HSV-1, the clinical manifestations are typically brief and often asymptomatic. However, the spread of HSV-1 can result in adverse complications, including stromal keratitis (HSK), genital herpes, and herpes simplex encephalitis (HSE) [1]. HSV-2 disease, on the other hand, is associated with higher morbidity, severity, and stigmatization, causing significant distress in affected individuals. Aside from causing recurrent, potentially ulcerative disease, HSV-2 increases the risk of HIV acquisition due to the persistence and enrichment of HIV susceptible cells in genital lesions [1, 8, 10, 15, 18]. Additionally, HSV-2 is regarded as a TORCH pathogen and can cause adverse effects in a fetus or newborn if transmitted during pregnancy. Unfortunately, a cure or vaccine is yet to be approved by the U.S. Food and Drug Administration, with many candidate vaccines failing to succeed in clinical trials [2,6]. Antiviral therapies, such as acyclovir, have been shown to reduce the frequency and intensity of outbreaks of herpetic lesions [2,6,8]. High worldwide prevalence, risk of adverse events, and the absence of an efficacious vaccine highlight the need to understand HSV pathology to develop effective and safe therapeutic interventions.

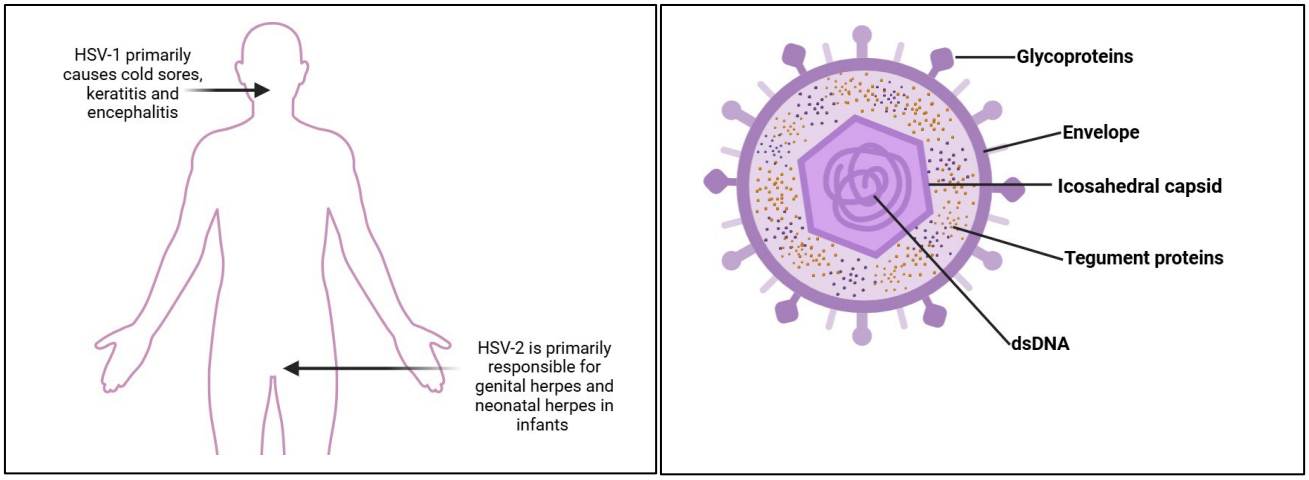

Figure 1 Sites of HSV Infection and Disease and Herpes Simplex Virus Structure

1.2 HSV Pathogenesis

The pathogenesis of human herpes virus disease depends on intimate personal contact of a susceptible host with an individual who is shedding HSV [1]. HSV initiates primary infection by gaining access to the basal layer of the epidermis of oropharynageal and genital mucosal tissue, a process that can be exacerbated by a broken skin barrier or open lesions [2]. Replication in the epithelium, particularly in basal keratinocytes, and subsequent cell-to-cell spread are accompanied by inflammation and tissue damage, manifesting as characteristic herpes blisters [1]. Following efficient replication in epithelial cells, HSV disseminates into the axons of sensory neurons in the epidermis and migrates retrogradely into the trigeminal root ganglia or sacral ganglia [1, 2,3]. HSV establishes a lifelong latent infection within neuronal cell bodies, where it is protected from immune detection. It is not entirely understood how the decision to enter latency is made, although studies suggest that the distinctive architecture of neurons, including axonal transport, is a determining feature [9, 48]. HSV intermittently reactivates in neurons, leading to viral lytic transcription and synthesis of virion proteins [48]. Viral capsids then travel anterogradely to axonal termini, where they mature and are released to infect epithelial cells [4, 48]. Known stimuli for viral reactivation include emotional and physical stress, UV exposure, and immune suppression [1, 9, 48]. Epithelial cells, including keratinocytes and dermal fibroblasts, are the major targets of HSV during productive infection [4, 20]. Recurrent disease is typically asymptomatic in immunocompetent individuals but may manifest as self-limiting lesions found primarily around the mouth, face, and eye (HSV-1) or genital area (HSV-1 and 2) [2, 9]. In general, while HSV-1 and 2 reactivate frequently, the immune system continually suppresses the duration and severity of the reactivation period [2, 9].

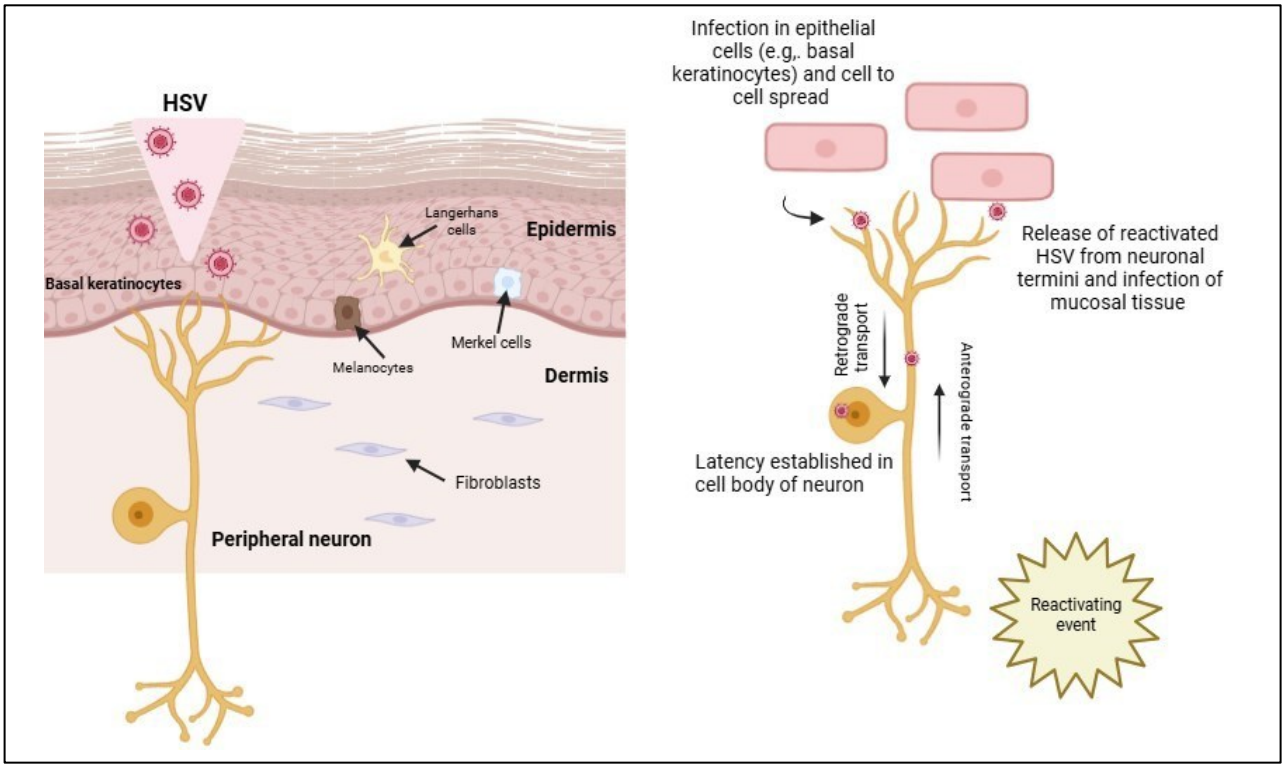

Figure 2 Stages of HSV infection HSV gains access to epithelial cells in the mucosa or epidermis typically through damage or abrasion to the skin barrier. HSV-2 primarily infects basal keratinocytes in the *stratum basale* layer of the epidermis and spreads cell-to-cell before reaching the nerve endings of peripheral neurons. HSV undergoes retrograde transport to the neuronal cell body, where it establishes latency. A reactivating event, such as UV damage, stress, or hormonal fluctuations, promotes viral anterograde transport to the nerve endings and subsequent release of the virus that can infect cells.

Shedding

HSV-2 disease is further complicated by frequent yet silent "shedding" during reactivation, contributing to its high seroprevalence worldwide [13]. "Shedding" refers to the detection of viral particles at mucosal sites and is commonly detected serologically [10, 13]. Differences in shedding patterns and clinical manifestations vary and are limited to observational studies, which can underestimate the true prevalence of these conditions. Available data have demonstrated that both HSV-1 and HSV-2 shedding decrease with time, and often in the absence of symptoms [10, 13]. Transmission can still occur during asymptomatic shedding, increasing the reservoir of infected individuals. Additionally, maternal shedding of either HSV-1 or HSV-2 at the time of birth poses a significant risk to a newborn; approximately 14000 infants are infected with HSV, with clinical outcomes ranging from noninvasive disease of the skin, eyes, and mouth to invasive systemic infection [1]. The antiviral drug acyclovir and related compounds dramatically decrease morbidity but do little to prevent permanent neurological deficits [1]. Improved seroprevalence surveillance would be insightful to physicians; in the meantime, suppressive therapy can reduce the probability of transmission.

Variability in disease manifestation

HSV-2 disease manifestation varies greatly and is poorly understood. Most affected individuals experience subclinical or asymptomatic reactivations [1]. In contrast, some individuals endure severe ulcerative disease despite fully competent immune systems.

Immunocompetent hosts generally experience self-limited HSV-2 mucosal ulcerations. While reactivations are frequent, they are equally rapidly resolved, with studies demonstrating viral clearance occurring within 12 hours of reactivation, with little to no clinical symptoms [13]. The lack of physical symptoms does not indicate a lack of infectivity; asymptomatic shedding and subsequent unintentional transmission allow HSV-2 to persist as a significant global burden.

Unfortunately, some individuals experience symptomatic episodes characterized by herpetic genital or oral lesions, which can also vary in severity [1, 13]. Moreover, complications such as recurrent meningitis, hepatitis, and pneumonitis can occur during the acquisition or reactivation of infection [13]. These complications typically arise due to poor immune control of the virus, which is common in individuals who have AIDS or have undergone recent organ transplantation or chemotherapy [13].

Prior studies have attempted to elucidate the cause of differential disease manifestation, with data highlighting differences in viral dynamics as a contributing factor to variations in host immune competency. One study determined that high viral load correlates with the development of genital ulcers [13]. Correlations between shedding patterns and disease severity suggest that the duration of the shedding period influences disease outcome, with prolonged genital shedding being associated with a higher initial viral load and a greater likelihood of symptoms and lesions [17, 30].

Other studies have focused on the competency of the host immune response as the determinant of disease severity. Rapid HSV clearance, reported in most asymptomatic individuals within 6-12 hours after shedding, illustrated prompt and effective host defense mechanisms [16]. This phenomenon is orchestrated mainly by HSV-specific CD8+ T cells, whose presence in genital lesions suggested that they either migrate rapidly to the site of replication or remain in the genital mucosa as immune surveyors of reactivation [22]. This discovery led to the establishment of HSV-2-specific CD8+ T cells as the persisting T cell population responsible for the localized and rapid clearance of HSV-2 reactivation, emphasizing the role of the peripheral mucosal immune system in controlling HSV-2 [22].

Vaccines

The overwhelming prevalence of HSV and its adverse effects on sexual, reproductive, and maternal-child health disparities make the development of a vaccine a public health priority. Currently, the U.S. Food and Drug Administration has not approved any HSV vaccine [6], and promising vaccine candidates have only shown partial efficacy in clinical trials. Acyclovir is the primary treatment for HSV infections, reducing the frequency and intensity of outbreaks of herpetic lesions, provided treatment begins in the early stages of the viral lytic cycle [6].

Several studies have incorporated chemokines and cytokines as adjuvants in vaccine development, demonstrating promising results in various murine models. The use of chemokines and cytokines primarily relies on their ability to recruit CD4+ and CD8+ T cells. The addition of IL-12 and IL-21 to an HSV-2 gD DNA vaccine has been shown to induce a strong T cell response following challenge with HSV-2 [77]. Chemokine-based adjuvants, such as CCL19 and CCL28, have been shown to enhance HSV-2-specific antibody responses, cytokine induction, and T cell enrichment in mouse spleens [78]. Incorporating an IL28B adjuvant enhanced humoral and T cell responses compared to a gD DNA vaccine alone [44]. A prime and pull method that involves "priming" with a vaccine, followed by recruitment of activated T cells through topical chemokines, has been shown to retain T cells and mediate protective immunity in mice [80]. Although these studies are awaiting clinical validation, the use of chemokine-based adjuvants is a promising avenue for vaccine development.

1.3 Immune Response to HSV: Innate and Adaptive Immune Responses

Innate Immune Response

The innate immune response to HSV involves recognizing the infection, producing Type I interferon (IFN), and orchestrating cellular defenses primarily through natural killer cells (NKs) and dendritic cells (DCs). Resident macrophages represent the first line of defense against HSV and rapidly respond to infection [58]. Shortly after, neutrophils are quickly recruited to sites of viral infection and contribute to phagocytic processes along with macrophages. Macrophages engulf and degrade HSV, exposing viral DNA to cytosolic DNA sensors and further activating innate signaling pathways. Pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), exacerbate HSV detection by recognizing foreign viral components and rapidly inducing signal transduction pathways to signal invasion. The major signaling pathway in primary HSV infection involves the induction of Type I IFN. Type I IFNs, discussed in detail below, are a subgroup of cytokines that interfere with viral propagation by eliciting antiviral gene expression in nearby cells. The collective activity of these gene products includes inhibiting viral protein expression, inducing apoptosis, and recruiting immune cells to sites of infection [79]. As a result, compromised tissues

assume an antiviral state that effectively minimizes viral replication and spread. Moreover, cell type-specific contributions are primarily attributed to natural killer cells (NKs) and dendritic cells (DCs) [1, 79]. NKs are potent cytotoxic lymphocytes that can directly eliminate HSV-2-infected cells by producing perforin, granzymes, and activating apoptotic pathways [79]. Furthermore, they produce IFNs, such as IFN-γ, that contribute to an overall antiviral state. Multiple studies have demonstrated that NK deficiencies correlate with more severe HSV disease outcomes [27]. Dendritic cells, particularly plasmacytoid dendritic cells (pDCs), constitute another critical cell type involved in the innate response to HSV. pDCs primarily produce Type I IFN, further enhancing antiviral signaling pathways [1, 79].

Unfortunately, HSV directly counteracts most of these processes, emphasizing their importance as viral antagonists. Ultimately, the innate immune response represents the first line of defense against HSV by directly eliminating and limiting viral spread or initiating and exacerbating a cytokine response to produce an effective antiviral state. These processes subsequently support inflammatory mechanisms that further enhance and recruit cells of the adaptive immune system.

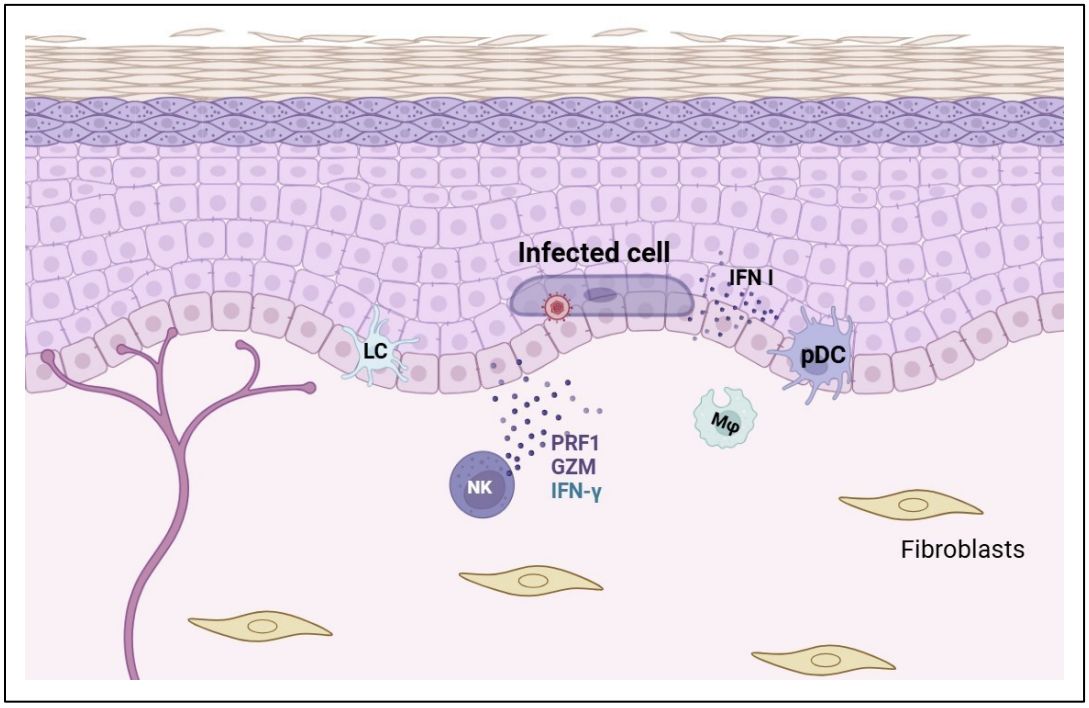

Figure 3. Key innate immune mechanisms involved in HSV-2 infection and resolution. The innate immune response to HSV infection involves pathogen recognition, antiviral signaling, and elimination of infected cells by key innate immune cells. Resident macrophages, neutrophils, and dendritic cells are heavily involved in the elimination of infected cells and immune signaling. Natural killer cells and dendritic cells produce Type I IFN, an antiviral cytokine that stimulates the expression of antiviral genes, inhibits viral replication, and mediates immune signaling.

Adaptive Immune Response

Despite the efficacy and vigor of the innate response, the adaptive immune response is essential for viral containment and clearance. During primary infection, the initial influx of CD4 T cells is crucial for producing interferon-γ (IFN-γ) and further activating CD8 T cells [2]. Cytotoxic CD8 T cells infiltrate HSV lesions later than CD4 T cells and are essential for HSV clearance and eliminating infected cells through direct and indirect killing mechanisms [2]. Upon recognizing HSV antigens on MHC class I molecules, activated CD8+ T cells release cytotoxic granules containing perforin and granzymes that induce the apoptosis of infected cells. Additionally, CD8+ T cells secrete TNF-α, TNF-β, and IFN-γ, contributing to the establishment of an antiviral state [2, 14, 20, 26].

Importantly, some CD8+ T cells remain in the dermal-epidermal junction (DEJ) after HSV clearance, acquiring a tissue-resident memory (TRM) T cell phenotype as dedicated surveyors of recurrent HSV [1,2,14]. Zhu et al. have well characterized this phenotype, verifying the tissue-resident memory T cell signature gene expression in HSV-2-positive tissue biopsies [26]. In particular, the downregulation of *CCR7*, *S1PR1*, and *KLF2* (lymphocyte egress) and the upregulation of *CD69*, *CD103*, and *CD49a* (lymphocyte retention) distinguish tissue-resident memory T cells from other circulating memory T cells [26, 17, 18, 19, 20]. Furthermore, TRM CD8+ T cell localization at the DEJ and proximity to sensory neurons allow for robust and rapid proliferation and elimination of the virus released from nerve endings [14,15]. In the early hours of reactivation, an HSV-2-specific TRM T cell can induce rapid contact-mediated apoptosis; however, mathematical models have demonstrated that TRM T cell contact-mediated killing is insufficient if the infection expands to hundreds or thousands of cells over 12 to 24 hours [15].

Another study by Zhu et al. revealed that, despite their prominent roles in viral clearance, the densities of CD8+ and CD4+ tissue-resident memory T cells are significantly lower than expected, and high densities are relatively uncommon [4]. To uncover how a small subset of resident T cells can effectively limit the rapid spread of HSV-2, in situ studies using human genital HSV-2 infection were analyzed to reveal immunological cross-talk between DEJ TRM CD8+ T cells and their neighboring epithelial cells [4]. These findings suggest that TRM cells must initiate a rapidly diffusing, polyfunctional cytokine response, in addition to cytotoxic killing of infected cells, to hinder viral spread [15, 19]. Cytokine secretion is essential for inducing intrinsic immunity in neighboring cells such as keratinocytes, which are highly susceptible to HSV-2 infection. Moreover, resident CD8+ T cell production of IFN-γ has been shown to correlate with viral load and enhanced innate resistance and is locally enriched in areas of reactivation [15, 19]. Epithelial cells neighboring resident CD8+ T cells displayed elevated antiviral signature expression, primarily related to IFN-γ

expression [20]. Keratinocytes demonstrated elevated levels of genes involved in antigen presentation (*B2M*, *HLA-A*, and *MICB*), chemoattraction (*CXCL10*, *CCL5*, and *CCL8*), and intracellular antiviral restriction (*IFITM1, IFITM2, IFITM3, MX1, MX2, ISG15, HERC5, STAT1, IFI16, TRIM22, BST2,* and *RIG-I*) [20, 30].

Despite CD8+ T cells being the dominant adaptive immune cell type that contributes to protection, CD4+ T cells also play a crucial role in local viral containment. While CD4+ T cells are found in the dermis at lower densities than CD8+ T cells, they can assume a tissue-resident memory phenotype, proliferate, and contribute to the cytokine alarm system via production of IFN-γ [81, 82].

Regarding humoral immunity, antibodies against HSV have been demonstrated to mediate protection primarily in murine models. In particular, administering anti-HSV antibodies reduced the frequency of clinical disease and viral titers at the site of infection in a murine model of infection [83]. Moreover, B cell-deficient mice are more susceptible to encephalitis and keratitis in an ocular model of HSV-1 infection [84]. Further clinical verification in humans is necessary to unveil the humoral contribution to HSV.

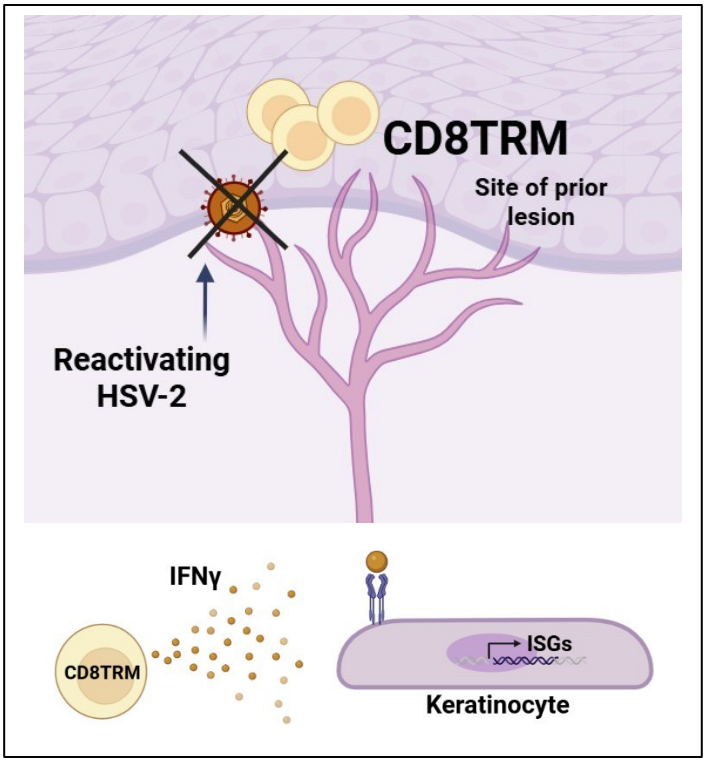

Figure 4. CD8+ tissue-resident-memory T cells control HSV-2 infection during reactivation. CD8TRM persist at the dermal-epidermal junction, in close proximity to innervating nerve endings and basal keratinocytes. During HSV-2 reactivation, cytotoxic CD8+ T cells rapidly eliminate virus released from nerve endings, limiting viral spread to epithelial cells. Moreover, CD8+ T cells are the main producers of IFN-γ, which can bind to neighboring, uninfected cells such as basal keratinocytes and induce the expression of antiviral genes. Induction of these interferon-stimulated genes enhances cell-intrinsic immunity and local protection against HSV-2 infection.

1.4 Role of Cytokines in Immune Response

While the innate and adaptive immune systems are essential for viral containment and clearance, they are primarily governed by cytokine-dependent mechanisms that facilitate the complex interplay between immune responses and the signaling networks that limit disease progression.

Cytokines are small, secreted proteins that include chemokines, interleukins, lymphokines, and monokines [40]. These proteins are released by immune and non-immune cells and may act in autocrine, paracrine, or endocrine actions on self, nearby, or distant cells, respectively [40]. Cytokine secretion and binding drive a variety of cell communication and signaling pathways within the immunoregulatory system. The main downstream effects of cytokine signaling include the upregulation or downregulation of immune cell activity [40]. During HSV primary infection and reactivation, cytokine signaling facilitates innate and adaptive immune responses through the recruitment, activation, and maintenance of key immune cells, as well as the induction of potent antiviral gene expression. Cytokines are likewise implicated in the exacerbation of inflammatory responses, leading to detrimental effects on the host and contributing to the pathogenesis of

disease. Conversely, cytokines represent attractive candidates for therapeutic intervention, due to their ability to facilitate immune cell activity.

Interferons

Interferons' biological function can be partially inferred from their etymology; the term interferon was derived from the observation that these soluble molecules “interfere” with viral replication [70]. IFNs are divided into three groups based on their affinity to specific receptors, structure, and function [70]. These include Type I IFNs, which encompass IFN-α, IFN-β, and IFN-κ; Type II IFNs, which include IFN-γ; and Type III IFNs, which include IFN-λ [48]. Most cells produce and respond to Type I IFNs, whereas IFN-γ is mainly produced by immune cells such as natural killer (NK) cells and T lymphocytes [28]. Epithelial cells are the primary source of Type III IFNs, but macrophages, monocytes, and dendritic cells also express the Type III IFN receptor [48]. The binding of IFN to its receptor activates signaling cascades that ultimately result in the transcription and expression of hundreds of IFN-stimulated genes (ISGs). The products of ISGs help establish an antiviral environment through contributing to inflammatory processes, apoptotic signaling, and cellular stress-response programs [28]. HSV has evolved various mechanisms to counteract interferon signaling [28]. Viral evasion and inhibition of the host interferon response may occur at any stage of interferon signaling, including during the viral surveillance and detection phase, as well as during the transcription, translation, and secretion of interferon, interferon-stimulated genes, and other antiviral factors [95].

Type I IFN

Type I IFNs include the well-defined interferon-alpha (IFN-α) and interferon-beta (IFN-β) along with interferon-kappa (IFN-κ), IFN-ϵ, and IFN-ω. These signaling molecules play a crucial role in innate immunity against viral infections by reducing viral replication and facilitating antiviral immune responses [49]. Type I IFNs are induced after pattern recognition receptors, such as TLR9, recognize pathogen-associated molecular patterns (PAMPs) [49]. Although many types of cells can produce IFN-α/β, circulating plasmacytoid dendritic cells (pDCs) primarily produce Type I IFNs, which bind to the dimeric interferon α/β receptor (IFNAR) to induce JAK/STAT signaling in infected or uninfected bystander cells. Subsequent signaling cascades lead to the transcription of interferon-stimulated genes, which inhibit viral replication at various stages of viral infection [50].

The induction of Type I IFNs is critical for establishing an appropriate innate immune response against HSV-2 infection. Type I IFN signaling promotes the recruitment and action of several inflammatory monocytes to the genital mucosa, including macrophages, neutrophils, and NK cells. Likewise, Type I IFNs help promote the Th1 adaptive immune

response. Loss of the Type I interferon signaling pathway increases susceptibility and vulnerability to HSV-2 infection. Moreover, Type I IFNs are vital immunomodulators of IFN-γ; impaired Type I IFN signaling impairs Type II IFN responses.

In addition to IFN-α and IFN-β, interferon kappa (IFN-κ) is a type I IFN expressed constitutively in keratinocytes and is induced significantly by other Type I IFNs and IFN-γ [75]. Studies have demonstrated that adding IFN-κ reduces HSV-1 replication in both differentiated and undifferentiated NHEK cell lines [75]. Conversely, HSV-1 and HPV inhibit IFN-κ gene transcription in human cervical keratinocytes, emphasizing the role of IFN-κ in antiviral defense [75].

Type II IFN (IFN-γ)

IFN-γ is the sole member of the Type II IFN family and the main cytokine of interest in this project. IFN-γ's pleiotropic effects involve macrophage activation, inflammation, host defense against intracellular pathogens, T-cell responses, tumor surveillance, and immunoediting [25]. IFN-γ is encoded by the IFNG gene, binds to the IFN gamma receptor (IFNGR), and is primarily secreted by natural killer (NK) cells and T cells, including CD4+ and CD8+ T cells [71].

IFN-γ Regulation and Signaling

IFN-γ production demands deliberate control to maintain homeostasis and elicit a competent immune response. Lack of an adequate IFN-γ response results in impaired immunity to respond to viral threats, while an excess cytokine response contributes to deleterious inflammation [24]. IFN-γ signals through interaction with its receptor, IFNGR, which is expressed on most cells, including immune cells, epithelial cells, and other tissue-specific cell types [47]. The receptor is composed of two subunits: IFNGR1, the ligand-binding alpha subunit, and IFNGR2, the signal-transducing beta subunit [203, 214]. The binding of IFN-γ with IFNGR results in a ligand-dependent receptor rearrangement and dimerization of IFNGR subunits. In turn, receptor-associated Janus Kinases JAK1 and JAK2 auto-phosphorylate and activate cytosolic transcription factor STAT1 via phosphorylation of its tyrosine at residue 701. Following phosphorylation, STAT1 homodimerizes and translocates to the nucleus. Here, it binds to GAS (gamma-activated sequences) elements in the promoter region and mediates the transcription of IFN-γ target genes. The products of IFN-γ signaling include immunomodulatory proteins and molecules that mediate a variety of cellular responses, including the activation and enhancement of immune cell function, as well as antiviral and antibacterial immunity, and the regulation of inflammation [31, 47, 48]. While canonical IFN-γ signaling leads to STAT1 activation, IFN-γ also has several non-canonical signaling pathways, including STAT3 [22].

Alternatively, IFN-γ has been shown to mediate STAT3 signaling. STAT3 is typically induced by IL-10, an anti-inflammatory cytokine [49]. By inhibiting STAT3, IFN-γ can suppress the expression of STAT3 target genes, including SOCS3, and ultimately antagonize the anti-inflammatory effects of IL-10 [49]. Moreover, STAT1 and STAT3 can cross-regulate and even compete with each other for the same receptor [51]. For example, STAT1 can be activated in the absence of STAT3 via a predominant STAT3 mechanism [51, 110]. While STAT1 and STAT3 have opposing biological functions, they have a shared regulatory mechanism that presents a nuanced source of immune variability.

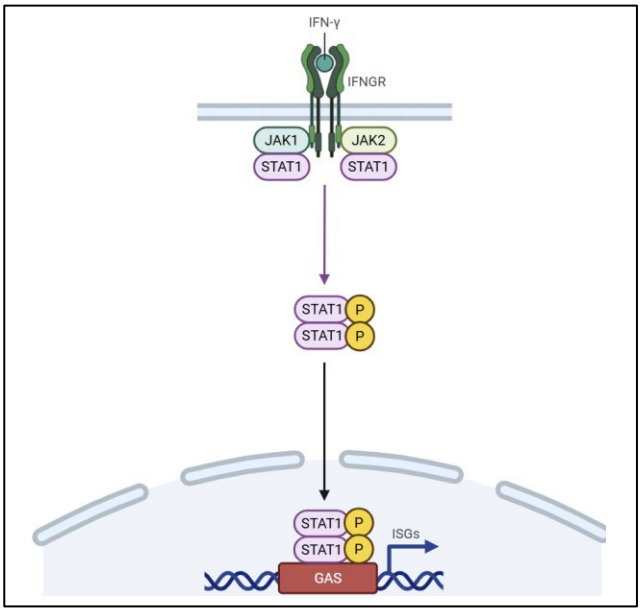

Figure 5: IFN-γ signaling through JAK/STAT IFN-γ signaling occurs through the JAK-STAT signaling cascade, resulting in the expression of IFN-γ-regulated genes. IFN-γ signaling cascade with the binding of IFN-γ to its receptor, IFNGR, which is composed of IFNGR1 and IFNGR2. Binding induces a conformational change that brings IFNGR1 and IFNGR2 closer in proximity. This allows for the recruitment of JAK1 and JAK2. IFN-γ binding induces the autophosphorylation and activation of JAK2, which phosphorylates JAK1. Phosphorylated JAK1 phosphorylates IFNGR1 at Y440, allowing STAT1 to bind. STAT1 is then phosphorylated by JAK2, dissociates from the receptor, and translocates to the nucleus. In the nucleus, pSTAT1 can interact with other transcription factors to induce the transcription of effector genes.

IFN-γ Role in Immunity

IFN-γ's immunomodulatory properties involve both innate and adaptive immune responses. IFN-γ plays a crucial role in host defense, immune surveillance, regulation of inflammation, apoptosis, cell cycle control, and the establishment of adaptive immunity. It is a critical cytokine in both the innate and adaptive immune response to herpes simplex virus infection. IFN-γ increases exposure and rapid recognition of infected cells by upregulating antigen processing and presentation, activates and recruits innate immune cells such as macrophages and NK cells, promotes an efficient T cell response, and mediates extensive gene expression programs that primarily involve cytokine and chemokine activities [70]. Additionally, IFN-γ is a critical factor in tumor surveillance and control. Its antitumor properties involve inducing tumor cell killing and recruiting effector cells to tumor microenvironments. While commonly involved in pro-

inflammatory and positive feedback loops, IFN-γ also exerts regulatory functions to limit tissue damage, such as selectively dampening Th1 cell responses via T regulatory cells [72]. Ultimately, this potent cytokine mitigates multiple branches of host immunity and defense while maintaining cellular homeostasis and immune resolution.

IFN-γ Role in HSV Disease

During the innate immune response to HSV, an early and potent Type II IFN response is necessary for proper innate cell activation and recruitment. For example, IFN-γ stimulates macrophage and natural killer cell activity, enhances antigen presentation, and triggers antiviral programs in cells via the production of Th1 cytokines and chemokines [51]. In particular, IFN-γ enhances macrophage nitric oxide production, promotes macrophage M1 polarization, and upregulates MHC class II expression on epithelial cells [51]. Additionally, studies have shown that early IFN-γ stimulation by NK cells is necessary for rapid dendritic cell maturation and migration in murine genital HSV-1 infection [51].

Likewise, IFN-γ plays a prominent role in the adaptive immune response and the development of immune memory to HSV-2. Type II IFNs are critical mediators of T-cell-mediated viral clearance. In addition to perforin and granzyme-mediated lysis of infected cells, CD8+ and CD4+ T cells produce IFN-γ [1]. A study by Johnson AJ et al. demonstrated that activated T cells, despite their specificity for HSV-2, were not protective against genital HSV-2 infection in the absence of IFN-γ signaling [52]. Another study demonstrated that CD4+ T cells require IFN-γ to enhance NK activation [38, 69].

IFN-γ-Induced Chemoattraction

In multiple HSV-2 infection models, IFN-γ elicits a robust chemokine response that recruits immune cells, including T cells, to the site of infection. For example, HSV-2 vaccination in mice has been shown to facilitate the retention of tissue-resident memory CD4+ T cells by IFN-γ-dependent macrophage production of CCL5 and CXCL10 [210]. Moreover, IFN-γ deficiency in HSV-2-infected individuals and mice has been observed to promote a Th2 cytokine response through IL-10 and IL-4, which dampen appropriate inflammatory processes necessary for viral clearance [11]. Type II IFNs are equally implicated in the B cell response to HSV-2; they are required for B cell recruitment, priming, and function during primary and secondary HSV-2 infection [51]. Recent studies have demonstrated the presence of naïve B cells and antibody-secreting cells in recurrent HSV-2 lesions, suggesting that B cells play a role in the resolution of reactivated HSV-2 lesions [53]. As such, IFN-γ has been shown to recruit memory B cells via the production of chemokines such as CXCL9 and CXCL10 [69].

IFN-γ-Induced Gene Expression

Through IFN-γ production, tissue-resident-memory CD8+ T cells instruct neighboring cells, such as basal keratinocytes, to express signature genes involved in chemotaxis, antigen presentation, and intracellular antiviral restriction [20, 26, 30]. The establishment of an antiviral state is critical for cellular resistance to viral replication and limiting the spread of the infection. During HSV-2 recurrent disease, the bulk of viral clearance that occurs has been shown to correlate with surges of IFN-γ, signifying the cytokine's contribution to viral elimination [1].

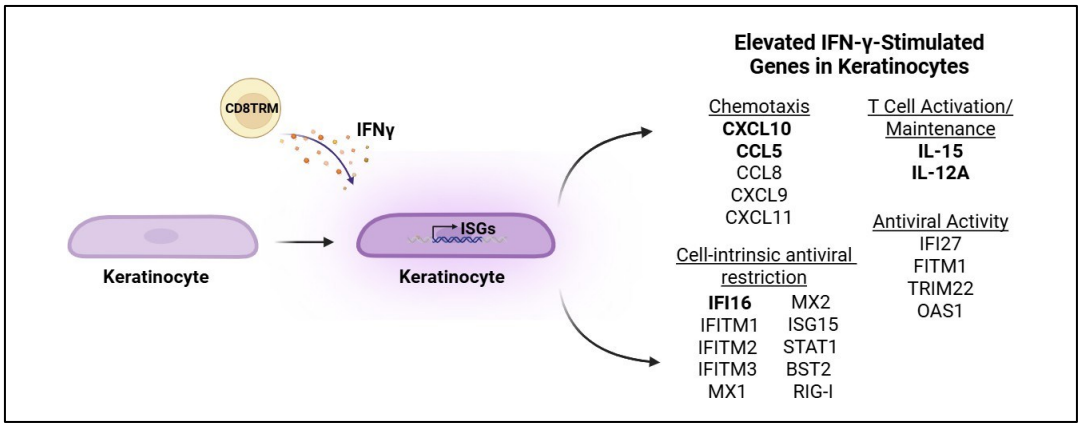

Figure 6: IFN-γ produced by CD8+ TRM induces antiviral gene expression in neighboring keratinocytes. ISGs include gene products responsible for chemotaxis and immune cell recruitment (*CXCL10, CCL5, CXCL9, CXCL11*), intracellular antiviral restriction (*IFI16, IFITM1, IFITM2, IFITM3, MX1, MX2, ISG15, HERC5, STAT1, RIG-I*), T cell activation, maintenance and retention (*IL-15, IL-12A*), antigen presentation (*B2M, HLA-A, MICB)* and other antiviral functions (*IFI27, FITM1, TRIM22, OAS1*). *These genes are implicit in tissue protection but only represent a subset of IFN-γ-induced genes.

IFN-γ Associated Pathologies

Dysregulated IFN-γ production has been associated with excessive tissue damage and necrosis with recurrent genital herpes [73]. Moreover, studies have emphasized the timing of a potent IFN-γ response, demonstrating that delayed signaling increases disease severity [73]. While IFN-γ has proven to be a key modulator in host cell immunity to herpes simplex virus, establishing its optimal potency and timing is key to extrapolating the best therapeutic approach to severe and asymptomatic HSV infection

Chemokines

Chemokines are small, structurally related proteins that mediate leukocyte trafficking and promote the maturation and resolution of immune responses [1, 40]. Chemokines are primarily produced by immune and structural cells in response to infection and bind to target cells that express their corresponding receptors [2]. Subsequent chemotactic pathways ensure that cellular traffic, including the migration of T cells to appropriate targets, is temporally and spatially regulated. Disturbing this complex yet fine-tuned system often leads to inflammatory complications [33].

During HSV-2 reactivation, immune, epithelial, and endothelial cells express chemokines and cytokines necessary for viral clearance [1,2]. The mobilization of effector cells, including T cells and NK cells, is dependent on chemokine expression. T cell-mediated expression of chemokines and cytokines has been shown to increase viral inhibition and clearance in HSV-2-affected skin [1, 2]. Resident dendritic cells (DCs) in the skin and mucosa and around reactivating neurons in the ganglia contribute to chemokine signaling and help activate immune cells [2]. Epithelial cells, such as keratinocytes, play a pivotal role in viral infection and are the primary target of HSV-2. Rapid and successful viral clearance heavily depends on keratinocytes' ability to produce chemokines that recruit T cells to the site of infection [20]. Additionally, chemokine signaling enables infected cells to communicate with neighboring cells, thereby establishing an antiviral state within the local tissue environment.

As mentioned above, chemokines have been increasingly used as adjuvants in murine HSV vaccine trials. Multiple vaccine programs have incorporated chemokines alongside DNA vaccines or topical applications to stimulate a potent inflammatory response, recruit immune cells such as CD4+ and CD8+ T cells, and support the activation and maintenance of T cells [2].

1.5 Cytokines of Interest in This Study

CXCL10, CXCL9, CXCL11, and CXCL8

CXCL10 is an IFN-γ-induced, pro-inflammatory chemokine that binds to the CXCR3 receptor expressed on activated Th1 cells, natural killer (NK) cells, dendritic cells, macrophages, and B cells [33]. CXCL10 primarily facilitates the recruitment of immune cells, such as virus-specific CD8+ T cells, and promotes a pro-inflammatory Th1 immune response to viral infection [32, 69]. This chemokine is strongly induced in response to many viral pathogens, including HSV-2, dengue virus, and HIV [69, 82]. During infection, IFN-γ stimulates CXCL10 secretion from various cells, including immune cells, epithelial cells, and endothelial cells. As a result, potent chemokine gradients recruit activated Th1 lymphocytes, monocytes, T cells, and NK cells to the site of infection [33].

In HSV infection, CXCL10 plays a critical role in the recruitment of T lymphocytes and NK cells to the site of infection [69]. Studies demonstrate that the absence of CXCL10 expression significantly alters the ability of the host to control genital HSV-2 infection [69]. Results from CXCL10 knock-out studies displayed reduced NK cell and virus-specific CD8+ T cell mobilization to HSV-2-infected tissue, which are crucial for viral suppression [69]. Aside from CXCL10 knockout studies in murine models, variability in CXCL10 expression in humans has not been thoroughly explored.

Differences in chemokine signaling might elucidate the mechanism behind differential disease outcomes in infected individuals.

Despite its critical role in immunological control of HSV-2, altered CXCL10 expression is implicated in multiple diseases involving infection, immune dysfunction, and chronic inflammation [32]. One common consequence of CXCL10 overexpression is the exacerbation of inflammation and associated tissue damage. More recently, the role of CXCL10 in cancer pathogenesis has been elucidated to involve tumor development, metastasis, and dissemination [32].

CXCL9 is a closely related chemokine that binds to the CXCR3 receptor, which is expressed on activated T cells, NK cells, monocytes, dendritic cells (DCs), and B cells. It is strongly induced in response to viral pathogens, including HSV-2, lymphocytic choriomeningitis virus, murine hepatitis virus, and vaccinia virus. Studies have demonstrated that CXCL9-deficient mice are more sensitive to HSV-2 infection than WT animals based on viral titer, inflammation, and mortality. Compared to CXCL10, CXCL9 expression is delayed in the vagina but rapidly elevated in the draining lymph nodes following infection [83]. Loss of CXCL9 in the spinal cord results in a reduction of NK cell and virus-specific CD8+ T cell mobilization and cytolytic activity, along with an elevation in viral titer and expression of CXCL1, CCL2, CCL3, and CCL5. Some studies have demonstrated that CXCL9 deficiency can be compensated by CXCL10 expression, which can preserve the recruitment of NK cells. Although both CXCL9 and CXCL10 signal through the CXCR3 receptor, CXCL10 deficiency confers greater susceptibility to HSV-2 infection, based on viral titers, inflammation, and mortality [82].

CXCL11 is another chemokine that binds to the CXCR3 receptor. CXCL11 is upregulated during HSV-2 infection and recruits immune cells, including activated T cells and NK cells, to the site of infection.

CXCL8, also referred to as Interleukin-8 (IL-8), plays a pivotal role in the acute inflammatory response by recruiting neutrophils to sites of infection. During HSV-2 infection, CXCL8 is produced primarily by cells neighboring the infection. Recent studies have shown that HSV-2 can inhibit CXCL8 production in infected cells through the viral vhs protein, which degrades host RNA [86]. CXCL8-mediated neutrophil recruitment can control viral replication; however, neutrophils have also been associated with excessive inflammation and tissue damage.

CCL5

CCL5, also referred to as RANTES, is both a pro-inflammatory chemokine and a homeostatic factor, constitutively expressed in many cell types [34]. CCL5 is selectively induced after HSV infection, particularly in macrophages and

fibroblasts, and binds to receptor CCR5 with high affinity [66]. As a broad chemotactic agent, CCL5 recruits various immune cells to the site of infection, including memory and effector T cells, NK cells, dendritic cells, and other immune cells implicated in inflammation [67].

Concerning HSV infection, CCL5 supports Th1 cell differentiation and attracts virus-specific T cells to the site of infection, playing a role in immune cell recruitment and subsequent viral clearance [68]. In an HSV-2 nasal vaccine trial, a murine study demonstrated that the CCR5-CCL5 chemokine pathway is required for the migration and retention of HSV-2-specific effector cells to vaginal mucosa [107]. Immunization with live attenuated HSV-2 enhanced CCL5 expression; contrastingly, CCL5 inhibition diminished the number of HSV-2-specific effector cells in the vagina [107].

Despite its protective role in HSV-2 infection, CCL5 and its receptor are regarded as poor prognosis signature markers in various cancer types, such as renal, prostate, breast, cervical, lung, and ovarian cancers [108]. Cancer cells or nonmalignant stromal cells have demonstrated elevated CCL5 levels, and overexpression of CCL5 has been shown to promote carcinogenesis and stroma genesis [108]. Although the role of CCL5 in various cancers is under investigation, studies suggest that chemokines, including CCL5, promote the autocrine growth, immune evasion, and migratory potential of cancer cells.

Moreover, viruses can exploit both chemokine receptors and their signaling pathways. For example, human cytomegalovirus (CMV) secretes a soluble chemokine receptor (pUL21.5) which binds selectively to CCL5 with very high affinity, blocking its interaction with receptor CCR5 [107]. Notably, HIV uses CCR5 and/or CXCR4 chemokine receptors to attach and infect immune cells [107].

IFI16

IFN-γ-inducible protein-16 (IFI16) is a member of the pyrin and HIN domain (PYHIN) that functions as an antiviral, intracellular DNA sensor [109]. IFI16 plays a crucial role in HSV-2 replication by interacting with viral dsDNA, preventing its uncoiling and epigenetically silencing HSV gene expression [1, 88]. Biopsy tissue acquired from HSV-2-affected individuals demonstrated elevated levels of IFI16 [20]. Importantly, IFN-γ has been shown to enhance IFI16-mediated viral gene suppression and replication in HSV-2 infection [20]. In addition to viral restriction, IFI16 can further interact with the stimulator of interferon genes (STING) pathway, inducing the production of antiviral cytokines such as IFN-β [65, 66, 109].

The HSV protein ICP0 counteracts the effects of IFI16 by targeting it for proteasome degradation, highlighting the protein's necessity as an antiviral molecule [65]. IFN can increase IFI16 induction, allowing the protein to circumvent viral ICP0 [57]. Interestingly, studies have demonstrated that a single mutation in the G allele of the IFI16 gene is associated with natural resistance to genital HSV-2 infection, emphasizing its potential as a target for therapeutic intervention [88].

Molecule	Role
CXCL10	IFN-γ induced chemokine; recruitment of T and NK cells Chemotaxis, induction of apoptosis, regulation of cell growth, and mediation of angiostatic effects
CCL5	Pro-inflammatory chemokine Chemotaxis
IL-15	Pro-inflammatory cytokine Activates and maintains NK cells and CD8+ T cells
IL-12A	Proinflammatory cytokine Stimulates the development of T helper 1 (TH1) cells
IFI16	Antiviral regulator, viral dsDNA sensor, strongly induced by IFN-γ
STAT1	Key factor in type I and II IFN signaling
STAT3	Key factor in type I and II IFN signaling
IFNGR1	Receptor for interferon-gamma (IFN-γ)

Table 1: Cytokines of interest in this project

IL-15

Interleukin-15 (IL-15) is an inflammatory, pleiotropic cytokine that plays a significant role in the activation and survival of CD8+ T cells, NKT cells, γδT cells, and NK cells. IL-15 is primarily secreted by dendritic cells and binds with high affinity to IL-15Ra on the same cell. IL-15 is then presented to T and NK cells, which activate JAK1/JAK3 along with STAT3/STAT5, PI3K/Akt, and Ras/Raf/MAPK pathways [105]. These signaling cascades trigger antiviral and inflammatory responses, as well as enhance anti-tumoral immune responses. Paradoxically, the overexpression of IL-15 can result in the development of hematological malignancies.

IL-15 is an essential component of innate antiviral immunity against HSV-2. NK cells, which contribute to antiviral protection, require IL-15 for development and activation. Multiple studies have demonstrated NK cell depletion following IL-15 knockouts, resulting in higher viral titers and increased mortality following HSV-2 infection [103, 104]. Moreover, IL-15 deficiency indirectly reduces IFN-γ production through NK cell depletion, thereby increasing susceptibility to HSV-2 infection [103, 104]. Studies suggest that the initial induction of IL-15 from infected epithelial

cells or macrophages in the submucosa leads to activation of NK cells, which can then provide innate protection against genital HSV-2 infection [103].

IL-15-based therapies have become more promising, especially in the field of cancer and HIV infection [105, 106]. IL-15's role as a growth factor for T and NK cells has been exploited to support adoptively transferred T cells and enhance the efficacy of tumor-targeting antibodies [105]. Moreover, IL-15 is an attractive alternative to IL-2 therapy due to its lower toxicity in vivo [106]. Overall, incorporating cytokines in vaccines and lymphocyte transfer can increase therapeutic efficacy.

IL-12A

IL-12A is a protein that heterodimerizes with IL-12B to form IL-12, a proinflammatory cytokine that signals through the IL-12 receptor (IL-12R) expressed on T, NK, and B cells. Binding triggers the activation of the JAK/STAT4 pathway [102]. IL-12 is primarily produced by phagocytic and antigen-presenting cells in response to pathogenic products [102]. Normal IL-12 signaling triggers the JAK/STAT4 pathway in target cells, resulting in potent Th1 responses, inhibition of Th2 responses, and overall inflammatory conditions [102].

HSV-2 has been shown to elicit IL-12 expression in macrophages, representing early cytokine production during viral infection [101]. In turn, IL-12 stimulates NK cell activity and induces the production of IFN-γ from NK and T cells, which are essential to control HSV-2 infection [37, 63, 102]. IL-12 is a dominant factor in IFN-γ production by T and NK cells and is required for optimal IFN-γ synthesis [100]. Likewise, IL-12 is strongly induced by IFN-γ, which mediates most of its immunological activities. Murine studies investigating HSV-2 infection demonstrated significant induction of IL-12, which was enhanced by IFN-γ [101].

IL-12 has been used effectively as an adjuvant in vaccine trials targeting cancer due to its ability to induce multiple inflammatory cytokines and stimulate natural killer (NK) cells, dendritic cells (DCs), and naïve T cells [102]. Conversely, IL-12's ability to promote a potent Th1 response has been shown to exacerbate inflammatory cell-mediated diseases such as inflammatory bowel disease (IBD), collagen-induced arthritis (CIA), and insulin-dependent diabetes mellitus (IDDM) [102].

1.6 Thesis Aims

Herpes simplex virus type 2 (HSV-2) is associated with highly prevalent and often recurrent ulcerative disease, ranging from asymptomatic, non-discernible illness to sporadic, severe manifestations [1]. Despite valiant efforts, the variability behind HSV-2 disease manifestation is poorly understood. HSV-2 prevalence is further associated with an increased risk of infection with human immunodeficiency virus (HIV), due to the influx of HIV-susceptible CD4+ T cells in ulcerative regions [15, 18]. As HSV can establish latency in sensory ganglia and reactivate throughout an individual's lifetime, a host's innate and adaptive immune system must continuously surveil and contain viral activity [1]. HSV clearance has been attributed to HSV-specific T cells that persist in the HSV-affected environment, and to the chemokines and cytokines that regulate the antiviral T-cell response [1,2,3,8]. In particular, interferon gamma (IFN-γ), can induce viral clearance through the activation of interferon-stimulated genes (ISGs) that encode antiviral proteins, microbicidal molecules, phagocytic receptors, chemokines, and cytokines [1,3,8]. We suspect that differential expression and regulation of ISGs contribute to the wide range of clinical outcomes observed in infected individuals. A thorough examination of IFN-γ-mediated gene expression may elucidate a source of variable HSV-2 prognosis and novel drug and vaccine targets to alleviate the burden of HSV [4]. The project includes two aims: (1) Measure the effect of IFN-γ on chemokine expression in relevant structural cell types associated with HSV-2 lesions and (2) assess whether differences in IFN-γ-induced chemokine expression contribute to differential clinical outcomes in HSV-2-affected individuals.

Aim 1: Investigate the time and dose-dependent response to IFN-γ in primary structural cells.

Subaim 1A: Determine the time and dose-dependent effect of IFN-γ on select chemokine expression in primary human cell lines from HSV-2 positive donors. IFN-γ has been shown to induce antiviral gene expression in neighboring cells within the herpes lesion microenvironment, most notably keratinocytes [20]. We will collect and treat primary human keratinocytes derived from HSV-positive donors to analyze the time and dose-dependency of IFN-γ-driven ISG expression. Using RT-PCR, we will perform transcriptional profiling of target genes to determine differential gene expression in HSV-2-positive individuals.

Subaim 1B: Compare the expression of cytokines of interest between keratinocytes and fibroblasts. Basal keratinocytes represent the primary target of HSV-2 infection and play a critical role in ISG signaling [20]. Fibroblasts, which lie deeper in the dermis, are susceptible to HSV-2 infection and implicated in the antiviral response. We want to determine whether IFN-γ-mediated gene expression differs amongst cell types. A transcriptional analysis of ISG expression in keratinocytes and fibroblasts could reveal whether IFN-γ confers a cell bias. Consequently, differential gene expression in response to IFN-γ could help elucidate the role of these cell types in antiviral signaling.

Aim 2: Assess IFN-γ-induced chemokine expression in HSV-2-affected individuals with asymptomatic disease versus severe, ulcerative HSV-2 manifestation: While studies have identified a library of IFN-γ-induced targets, variable responses to IFN-γ are not well understood. Transcriptional profiling of HSV-2 positive donor-derived cells, including cells from asymptomatic donors and donors with frequent lesion development, will allow us to directly compare differential gene expression in response to IFN-γ. Ultimately, we want to investigate the correlation between IFN-γ-induced gene expression and HSV-2 disease severity

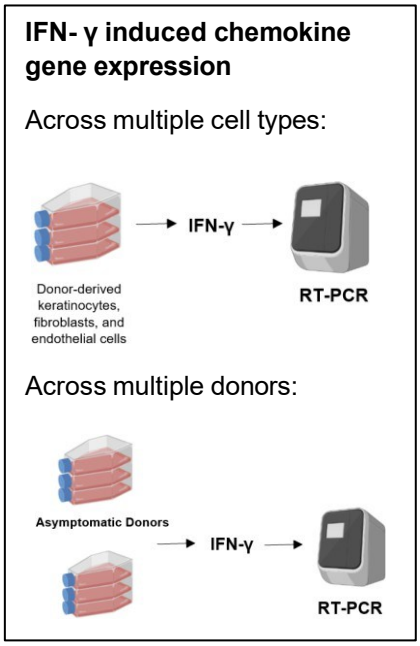

Figure 7: Thesis Aims

Chapter 2. Methods

Study participants and biopsy collection

Healthy, HSV-2 seropositive, and HIV seronegative adults were recruited by the University of Washington Virology Clinic in Seattle, Washington. The study protocol was approved by the University of Washington Human Subjects Review Committee, and written consent was acquired from all participants. Biopsy tissues were extracted from the arm, and biopsy procedures were carried out according to established methods. The biopsy disaggregation protocol was as follows:

Biopsy samples were removed from original tubes and washed gently in cold PBS. Samples were then submerged in cold Dispase I solution (Sigma D4818-2MG) and incubated overnight at 4°C. The next day, biopsy samples were removed from Dispase I solution and placed into PBS. Epithelium was gently removed from the dermis using tweezers and placed into a 0.6 mL tube containing 0.05% trypsin to dissociate keratinocytes (Gibco 25200-056). Epithelium samples were incubated at 37°C for 15 minutes. The separated dermis was placed in endothelial growth medium (Lonza EMB-MV2). Dermis was gently squeezed and massaged with curved tweezers to release endothelial cells. Dermis was then removed from the endothelial media and placed into an empty well on a 6-well plate, cut into two portions, and allowed to dry. Endothelial cells left in endothelial media were mixed gently, and 50 μL of cell suspension was added to each well of a 48-well plate. Endothelial wells were brought up to a total volume of 0.5 mL with endothelial media. At this time, the epithelium was collected and, using a blunt-tipped needle, keratinocytes were disaggregated in trypsin until the epithelium disk became almost entirely transparent. The trypsin solution was then neutralized with 5 mL of Q media before spinning down keratinocytes for 5 minutes at 200g. Keratinocytes were then collected and resuspended in 3 mL DermaCult (StemCell Technologies) in ROCK inhibitor before being seeded into a six-well collagen-treated plate. Keratinocyte and endothelial cell plates were spun at 350 rpm for 3 minutes. 3 mL of fibroblast media (PromoCell) was added to wells containing partially dry adhered dermis, and all cell plates were incubated at 37°C. Cells were fed every other day until the first passage.

Participant	Subject ID	Subgroup	Age at Enrollment	Sex	HSV Status
1	CH15172	Asymptomatic	63	F	HSV-1&2
2	KV13574	Asymptomatic	67	F	HSV-1&2
3	SF14604	Asymptomatic	56	F	HSV-1&2
4	LS14140	Asymptomatic	48	F	HSV-2
5	TN15060	Asymptomatic	44	F	HSV-1&2
6	RH16031	Severe	41	M	HSV-1&2
7	AL13135	Severe	74	F	HSV-1&2
8	SH14981	Severe	64	F	HSV-1&2
9	AW15979	Severe	40	F	HSV-2
10	MK13736	Severe	48	F	HSV-1&2
11	SA12604	Severe	59	F	HSV-1&2

Table 2: Subject demographics

Primary cell culture and viral strains

Primary donor-derived keratinocytes were isolated from skin biopsies and cultured in DermaCult Basal Medium (Stemcell Technologies 100-0501) and supplemented with DermaCult expansion supplements (Stemcell Technologies 100-0502), hydrocortisone (Stemcell Technologies 07925), and amphotericin B (Gibco 15290-019). Primary donor-derived fibroblasts were isolated from skin biopsies as described above and cultured in CnT-Prime Fibroblast Proliferation Medium (CellnTec CnT-PR-F). Keratinocytes were passaged using trypsin 0.05% (Gibco 25200-056) and neutralized using Q media in 10% FBS DMEM supplemented with penicillin and streptomycin. Fibroblasts were passaged using TripleE (Gibco 12604-013). All keratinocyte and fibroblast cultures were used at low passage (less than 5 for keratinocytes, less than 7 for fibroblasts). HSV-1 recombinant strain K26 containing the VP26-GFP fusion gene (2) and the HSV-2 wildtype strain 186 were used to infect cells.

IFN-γ Treatment

Interferon gamma (IFN-γ, Thermo/PeproTech 300-02-100UG) was resuspended as a stock solution at 100,000 U/mL (10μg/mL) in PBS supplemented with 0.1% bovine serum albumin (Sigma B4287) and stored at -20°C. Primary human keratinocytes were seeded into 12-well plates at 100,000 cells per well, then allowed to incubate overnight. The next morning, IFN-γ was serially diluted in media to generate working solutions of 0.01, 0.1, 1.0, 10, or 100 U/mL. Keratinocytes were left untreated as a control or treated with specified amounts of IFN-γ for 2, 4, or 24 hours. All experiments were completed in biological triplicate.

Viral Infection (Monolayer)

Primary keratinocytes were mock-treated or pretreated with IFN-γ at 0.1, 1.0, 10, or 100 U/mL for 0, 4, 8, 16, or 24 hours before infection. Cells were mock-infected or infected with the HSV-2 wildtype strain 186 at MOI = 1 for 4 hours. Triplicate samples were generated for each condition.

RNA Extraction and cDNA Synthesis

Following treatment with IFN-γ or infection with HSV, cells were directly lysed in their wells using QIAGEN RNeasy RLT buffer with 1% 2-Mercaptoethanol (QIAGEN 74104). Lysate was further homogenized using QiaShredder Mini Spin Columns (QIAGEN 79654). Samples were loaded onto RNeasy mini columns for RNA isolation per the manufacturer's protocol (QIAGEN 74104). Purified RNA was stored at -80 °C or immediately synthesized into cDNA following the SuperScript IV Primary Strand Synthesis protocol (Thermo/Invitrogen 18091050). cDNA was stored at -20°C.

RT-PCR

cDNA amplification was performed using TaqMan Universal Mastermix II (Thermo/Life Technologies 4440048), one microliter of cDNA, and probes for CXC10, CCL5, IL-15, IL-12A, IFI16, and beta-actin (Thermo). Samples were run in technical duplicates. The average Ct value between sample technical replicates for each probe was used to calculate ΔCt and ΔΔCt with a maximum Ct threshold of 35. Fold change in gene expression was expressed relative to mock expression levels and with respect to beta-actin, the housekeeping gene used in this study. Graphs were constructed using GraphPad Prism version 10.2.1.

Protein Analysis

Luminex assay was performed by the immune monitoring core to measure protein levels of CXCL9 and CXCL10 in cell culture supernatant. Primary keratinocytes were cultured and allowed to reach confluency before being seeded at 100,000 cells/well of a collagen-treated 12 well-plate. The next day, cells were mock-treated or treated with IFN-γ at 0.1, 1.0, or 10 U/mL for a total of 8 hours. Another set of primary keratinocytes was infected or pretreated with 10 U/mL of IFN-γ 4 hours before HSV-2 186 infection. To study the impacts of HSV-2 on cytokine production, IFN-γ-treated keratinocytes were either infected 2, 1, or immediately before IFN-γ treatment with an MOI of 1. Separate pools of keratinocytes were also infected 4 hours after treatment with IFN-γ. All treatment groups were exposed to a total of 8 hours of IFN-γ,

regardless of infection timing. 80 uL of supernatant was collected into separate wells of a 96-well plate after 8 hours for each experimental group to measure protein levels.

Immunofluorescence Staining

Primary human keratinocytes were cultured in DermaCult Basal Medium (Stemcell Technologies 100-0501) and seeded at 10,000 cells/well in 16-well chamber slides. Upon confluency, cells were mock-treated or treated with 0.01, 0.1, 1.0, 10, and 100 U/mL IFN-γ for 30, 60, 120, or 240 minutes. Cells were fixed in 4% formaldehyde (Boster Bio AR1068) in PBS for 15 minutes at room temperature. Following a wash in PBS, cells were incubated with wheat germ agglutinin (WGA) for 10 minutes at room temperature. Cells were permeabilized and incubated in 100% methanol at -20C for 10 minutes. Samples were blocked using a blocking buffer (0.3% Triton X-100, Acros 327371000, and normal donkey serum, Jackson Labs 017-000-121, in PBS). Primary antibodies in antibody buffer (0.3% Triton X-100 and 1% BSA in PBS) were applied to the slides and incubated overnight at 4C. Primary antibodies were used at the following dilutions: Tyr701pSTAT1 (1:800, Cell Signaling Technology) and Tyr750pSTAT3 (1:320, Cell Signaling Technology). The next day, cells were rinsed in PBS and incubated with AlexaFluor-conjugated secondary antibodies (Invitrogen) diluted at 1:200 in antibody buffer for 2 hours at room temperature. Following another rinsing step with PBS, stained cells were covered with SlowFADE Diamond with DAPI to stain nuclei (Thermo 536964) and imaged using Nikon Eclipse Ti2. All image analysis was completed using the Nikon Eclipse software suite.

Organotypic culture

Transwell cultures were generated using donor-derived fibroblasts for the dermis equivalent and donor-derived keratinocytes for the epidermis equivalent. Primary fibroblasts cultured in fibroblast media (PromoCell) were used to assemble the dermal equivalent. Dermis equivalents were generated on ice by mixing rat tail collagen (final concentration 4mg/mL) with ⅒ volume 10X M199 media, then neutralizing to a visual pH 7.0 using 1N NaOH. Immediately following neutralization, fibroblasts were resuspended in FTAL5 and mixed with the collagen solution for a final concentration of 6.25e5 cells per mL and embedded into a collagen mix (4mg/mL) with 10X M199 (Thermo) and 1N NaOH at >1.5e6 cells per 24-transwell plate. 100 µL of fibroblast collagen mix was aliquoted on the bottom of each transwell insert and allowed to solidify. The dermis was then submerged in FTAL5 media (CELLnTEC) for 24 hours. The next day, 200 uL of keratinocytes in FTAL4 media was added to the top of each transwell at 60,000 cells/well to form the epidermis equivalent. The culture model was allowed to grow for 3 days in submerged conditions before being lifted to the air-liquid interface and 14 days to form a complete stratified epithelium.

IFN-γ Treatment and Infection of Organoid Cultures

After formation of a complete stratified epithelium (14 days after being lifted to an air-liquid interface), transwell cultures were mock-treated or treated basolaterally with IFN-γ at 0.1, 1.0, 10, or 100 U/mL for 0, 2, 4, 6, 8, or 24 hours before collection. A set of transwell cultures was also infected with 2.5×10^5 PFU of HSV-2 wild-type strain 186 apically before using a 26G needle to wound the epithelium for viral entry. Organoid samples were collected using lysis buffer RLT and βME before RNA extraction.

Organotypic Culture Immunohistochemistry

Organotypic cultures were collected or treated 14 days post lifting to air-liquid interface, fixed in 4% formaldehyde for 1 hour at room temperature, and embedded in Cryo-Molds in O.C.T. (Electron Microscopy Sciences). Embedded tissues were kept at -80 °C until ready to be sectioned into 10 μM thick sections and placed onto standard Superfrost Plus slides (Fisher 12-550-15).

Slides were permeabilized and blocked for 30 minutes at room temperature using 1% Triton X-100 and 10% normal donkey serum in PBS. Primary antibodies diluted in antibody buffer (PBS containing 0.1% Tween-20 and 10% normal serum) were applied and incubated on slides for 1 hour at room temperature. Primary antibody dilutions were as follows: CK10 (1:1000 Abcam ab76318) and CK14 (1:2000 Abcam ab7800). Fluorescently conjugated secondary antibodies were diluted at 1:200 in antibody buffer and incubated on the slides for two hours at room temperature. Slides were mounted using Hoechst for 10 minutes to stain nuclei. Imaging was completed using a Nikon Eclipse Ti and image analysis was completed using Nikon Elements analysis software.

Organotypic Culture RT-PCR

Organotypic cultures were lysed and collected using lysis buffer RLT and βME. Tissues were disrupted and lysed using single-use plastic pestles (Sigma) then homogenized using QiaShredder tubes (Qiagen 79654). After homogenization, samples were transferred to new Eppendorf tubes and incubated at 55C in water and Proteinase K for 10 minutes. An equal volume of 100% ethanol was added to each sample before transferring samples to RNeasy spin columns for RNA extraction per the manufacturer's protocol (RNeasy Fibrous Tissue Kit Qiagen 74704). Purified RNA was stored at -80C or immediately synthesized into cDNA following SuperScript IV Primary Strand Synthesis protocol (Thermo/Invitrogen 18091050). cDNA amplification was performed using TaqMan Universal Mastermix II (Thermo/Life Technologies 4440048), one microliter of cDNA, and probes for CXCL10 and beta-actin (Thermo). Samples were run in duplicates.

The average Ct value between sample technical replicates for each probe was used to calculate ΔCt and ΔΔCt with a maximum Ct threshold 35. Fold change in gene expression was expressed relative to mock expression levels and with respect to beta-actin, the housekeeping gene used in this study. Graphs were constructed using GraphPad Prism version 10.2.1.

Statistical Analysis

Linear mixed models and unpaired two-sample Welch's t-tests were used to analyze differences in expression levels of interferon-stimulated genes amongst experimental groups of differentially treated donor-derived keratinocytes. Additionally, two-sample t-tests and linear mixed models were used to examine the differences between cohorts of individuals with asymptomatic and severe HSV-2 disease. *P* and *n* values are indicated in the text and Figure legends. Data analysis and presentation were performed using GraphPad Prism version 10.2.1.

Chapter 3. Data and Results

3.1 Donor-Dependent Keratinocyte Response to IFN-γ Treatment

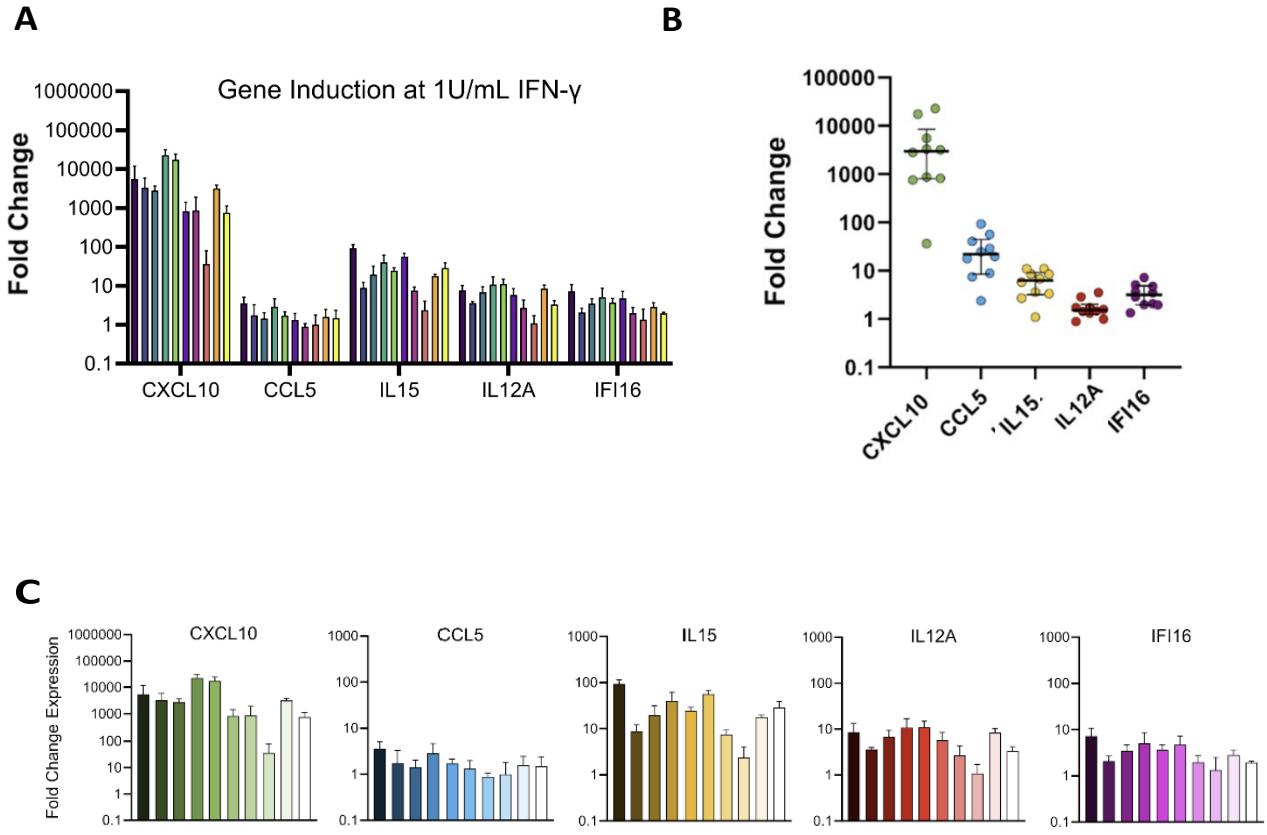

Figure 8 IFN-γ treatment of keratinocytes and transcriptional analysis of gene expression (A) Donor-dependent gene induction of *CXCL10*, *IL-15*, *IL-12A*, *CCL5,* and *IFI16* in response to 1.0 U/mL IFN-γ in primary keratinocytes from HSV-2 positive donors (n = 30). Primary human keratinocytes were cultured and treated with 1.0 U/mL IFN-γ. All cells were collected 4 hours post-treatment and run through quantitative RT-PCR. Target gene expression relative to matched mock-treated cells and normalized to β-actin. **(B)** Collective fold change and overall distribution of IFN-γ induced gene expression in primary cultured human keratinocytes following 1.0 U/mL IFN-γ treatment. Box and whisker plots represent median fold change, first and third quartiles and minimum and maximum fold change in gene expression (n = 10). **(C)** Individual gene induction profiles for *CXCL10, IL-15, IL-12A, CCL5,* and *IFI16*.

Average Ct										
	D1	**D2**	**D3**	**D4**	**D5**	**D6**	**D7**	**D8**	**D9**	**D10**
CXCL10	23.0	24.2	21.2	21.4	20.8	26.2	26.2	29.2	24.0	25.9
CCL5	33.0	32.5	33.1	34.0	32.1	34.4	35.0	31.3	33.0	34.8
IL15	26.5	25.9	25.9	25.9	24.0	26.0	27.7	30.5	26.0	28.5
IL-12A	29.3	28.9	27.8	29.9	28.9	28.8	31.0	32.3	28.8	31.3
IFI16	22.3	21.7	21.4	22.0	20.8	22.5	22.7	22.7	21.9	23.8
β-Actin	21.4	20.1	20.7	21.2	20.0	20.6	21.6	17.4	21.4	22.0
ΔCt = Ct(gene of interest) - Ct(housekeeping gene)										
	D1	**D2**	**D3**	**D4**	**D5**	**D6**	**D7**	**D8**	**D9**	**D10**
CXCL10	1.6	4.1	0.5	0.1	0.7	5.6	4.6	13.8	2.7	3.9
CCL5	12.3	14.0	12.4	12.7	12.1	14.2	13.4	13.9	11.6	12.9
IL15	5.1	5.8	5.3	4.7	3.9	5.4	6.1	13.1	4.7	6.5
IL-12A	7.9	8.8	7.1	8.7	8.9	8.2	9.4	15.0	7.4	9.3
IFI16	0.9	1.5	0.7	0.8	0.8	1.9	1.1	5.4	0.6	1.7
β-Actin	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
ΔΔCt = ΔCt(gene of interest) - ΔCt(reference sample)										
	D1	**D2**	**D3**	**D4**	**D5**	**D6**	**D7**	**D8**	**D9**	**D10**
CXCL10	-11.7	-11.0	-11.4	-14.4	-14.0	-9.4	-9.0	-3.5	-11.6	-9.4
CCL5	-1.7	-0.4	-0.4	-1.3	-0.8	-0.3	0.2	0.3	-0.5	-0.4
IL15	-6.5	-3.1	-4.1	-5.2	-4.6	-5.8	-2.9	-0.8	-4.2	-4.8
IL-12A	-2.9	-1.8	-2.7	-3.3	-3.4	-2.4	-1.3	0.0	-3.1	-1.7
IFI16	-2.7	-1.0	-1.7	-2.1	-1.8	-2.1	-0.9	-0.1	-1.5	-1.0
β-Actin	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Fold Change [2^-ΔΔCt]										
	D1	**D2**	**D3**	**D4**	**D5**	**D6**	**D7**	**D8**	**D9**	**D10**
CXCL10	5501.5	3288.9	2802.0	22733.4	17437.0	822.0	863.2	36.4	3162.8	753.5
CCL5	3.5	1.7	1.4	2.9	1.7	1.3	0.9	1.0	1.6	1.5
IL15	93.0	8.8	19.5	40.4	24.6	56.4	7.5	2.4	17.8	28.9
IL-12A	8.6	3.6	6.8	10.9	11.0	5.8	2.7	1.1	8.5	3.3
IFI16	7.2	2.1	3.5	5.1	3.7	4.8	2.0	1.3	2.9	2.0
β-Actin	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

Table 3 Average Ct, ΔCt, and ΔΔCt used to calculate fold change in gene expression for each donor. Ct values were acquired from qPCR and represent the average Ct value of 3 biological replicates run as technical duplicates. ΔCt was calculated by subtracting the average Ct value of the gene of interest from the housekeeping gene average Ct (β-Actin). ΔΔCt was calculated by subtracting the reference sample ΔCt (average ΔCt of the corresponding untreated sample). Fold change was determined using ΔΔCt.

Keratinocytes are a major structural component of skin and mucosa, forming the epidermis that interacts with fibroblasts in the dermis and innervating sensory nerve endings at the dermis-epidermis junction (DEJ). Importantly, keratinocytes are the primary targets of productive HSV-2 infection. HSV-2 positive individuals experience a spectrum of disease outcomes, ranging from asymptomatic viral reactivation to severe ulcerative disease. IFN-γ released by DEJ CD8 tissue-resident memory (TRM) cells has been demonstrated to induce the transcription of antiviral genes in neighboring keratinocytes. We wanted to assess whether keratinocytes acquired from HSV-2-positive individuals of varied disease outcomes demonstrate differential gene expression in response to the same dose of IFN-γ.

Using donor-derived primary human keratinocytes, we performed a transcriptional analysis of a subset of IFN-γ-stimulated genes to investigate the donor-dependent keratinocyte response to IFN-γ treatment. We compared the differential expression of a subset of known IFN-γ-stimulated genes, including *CXCL10*, *IL-15*, *IL-12A*, *CCL5*, and Interferon-gamma-inducible protein 16 (*IFI16*), which have been implicated in immune cell recruitment, T cell activation and maintenance, and cell-intrinsic antiviral response.

Primary keratinocytes were isolated and cultured from skin tissue obtained from 10 HSV-2-positive individuals with variable disease severity. Primary keratinocytes were treated with 1.0 U/mL IFN-γ and collected 4 hours post-treatment. Preliminary data have shown that optimal gene induction occurs at 4 hours post-treatment.

Transcriptional analysis revealed variable, donor-dependent induction of target genes following IFN-γ treatment (**Figure 8A**). All donor-derived keratinocytes demonstrated augmented gene expression compared to baseline levels of select genes (**Figure 8A**). *CXCL10*, which encodes a chemoattractant implicated in T cell recruitment, was the most induced gene in response to IFN-γ (**Figure 8B, C**) and was significantly induced in all donors ($P < 0.001$ for all genes except for *CCL5,* $P < 0.05$) (**Figure 8A, C**). Despite this shared feature, donors demonstrated considerable variability and a spread in CXCL10 induction, ranging from a 2,700-fold change to an 85,000-fold shift in gene expression (**Figure 8A, 8B**). *IL-15* was the second most induced gene with an average fold change in gene expression of 29.9. *IL-12A* was the third most induced gene with an average fold change in gene expression of 6.13. *CCL5* and *IFI1,* responsible for immune cell recruitment and intrinsic antiviral defense, respectively, were the least induced in response to IFN-γ (**Figure 8B**). Importantly, *IFI16* baseline expression in keratinocytes is the highest out of all gene targets analyzed (**Table 3**).

Despite variability in induction amongst donors, our analysis reveals common patterns of gene expression shared amongst donors. *CXCL10* was induced the most (average 5740.1-fold change), followed by *IL-15* (29.9) and IL-12A

(6.13), while IFI16 (3.45) and *CCL5* (1.76) were moderately induced in response to the same IFN-γ dose (**Figure 8B**). This provides essential insight into predicting expected induction at a given IFN-γ dose. The dramatic induction of *CXCL10* suggests that this gene plays a significant role in antiviral defense, while other moderately induced genes are either less sensitive to IFN-γ, engage in different signaling pathways, are biologically active at lower concentrations, or play a minor role in antiviral defense in keratinocytes. Moreover, our analysis demonstrates that IFN-γ-mediated gene expression is highly variable amongst donors, suggesting that keratinocyte sensitivity to IFN-γ and subsequent differences in gene induction play a role in the magnitude and effectiveness of an IFN-γ-induced antiviral state in response to viral replication.

3.2 Dose-response analysis reveals dramatic upregulation of CXCL10 at low doses of IFN-γ

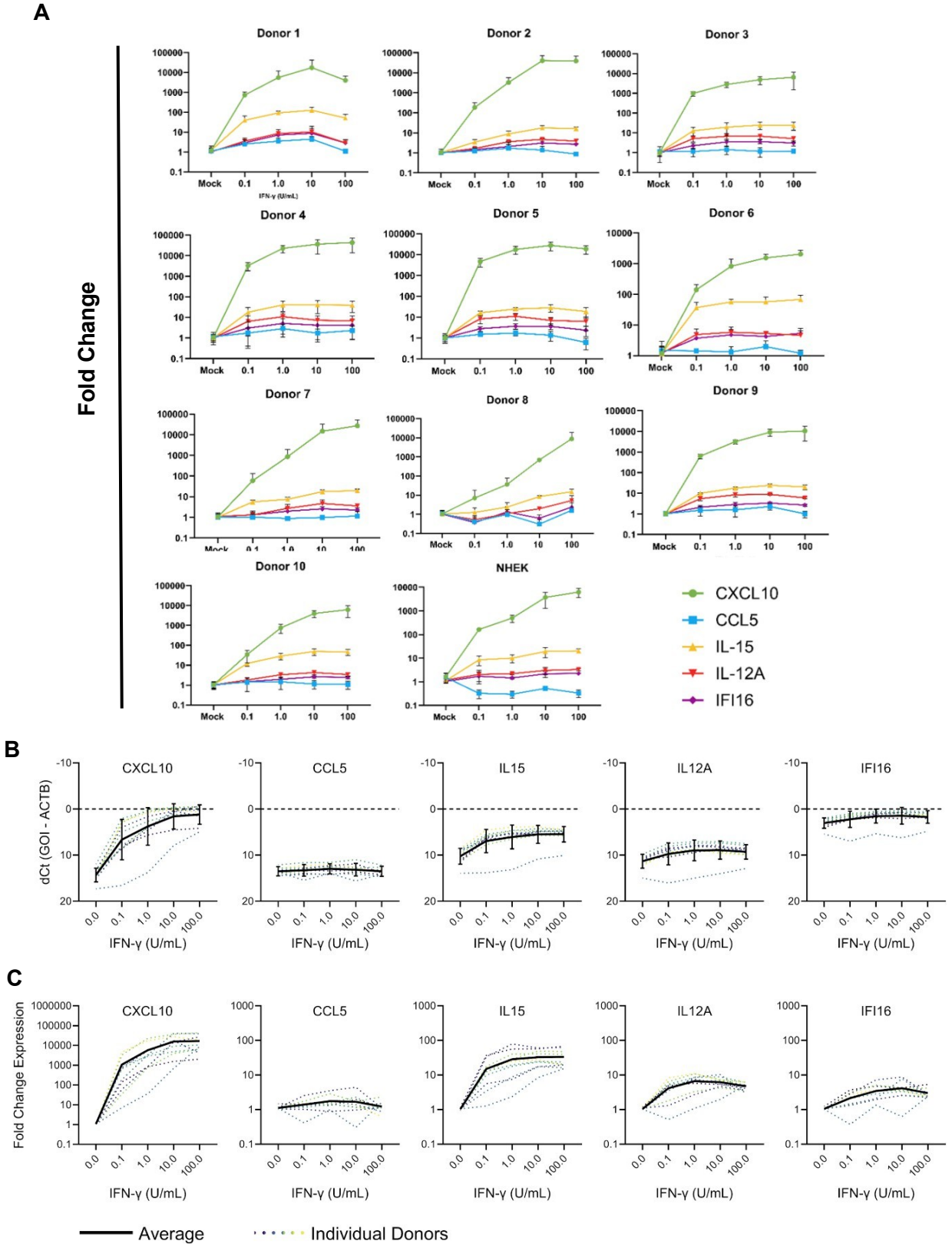

Figure 9 Primary keratinocytes demonstrate dose-dependent IFN-γ-induced gene expression. (A) Individual donor dose response curves demonstrating induction of *CXCL10* (green), *CCL5* (blue), *IL-15* (yellow), *IL-12A* (red), and *IFI16* (purple)—across varying concentrations of IFNγ (0.1 to 100 U/mL). Each subplot corresponds to a different donor, showing individual variation in gene expression responses. CXCL10 exhibits the highest induction across all donors, while other genes show moderate increases with IFN-γ stimulation. **(B)** Induction of *CXCL10*, *CCL5, IL-15, IL-12A,* and *IFI16* expression in primary keratinocytes (n = 10) following mock-treatment or treatment with 0.1, 1.0, 10, or 100 U/mL IFN-γ and normalized to β-actin (dashed line). Data is represented as dCt, which is calculated by subtracting the Ct value of induced genes from β-actin Ct values. All Ct values were acquired directly from qPCR with a maximum Ct threshold of 35. **(C)** Fold change in *CXCL10*, *CCL5, IL-15, IL-12A,* and *IFI16* expression in primary keratinocytes (n = 10) following mock-treatment or treatment with 0.1, 1.0, 10, or 100 U/mL IFN-γ. Fold change calculated as relative to mock expression normalized to β-actin. The bold line represents the average fold change expression across all donors. Dashed lines represent fold change expression in individual donors.

We next evaluated whether IFN-γ elicits dose-dependent gene induction in primary human keratinocytes. To determine how sensitive keratinocytes are to IFN-γ, we compared mock-treated primary keratinocytes to keratinocytes treated with 0.01, 0.1, 1.0, 10, or 100 U/mL of IFN-γ. We then performed a transcriptional analysis of *CXCL10, CCL5, IL-12A, IFI16,* and *IL-15* described above.

Little to no induction of gene expression was detected at 0.01 U/mL of IFN-γ, suggesting a threshold for the minimum IFN-γ dose required to induce transcription of target genes. Compared to the housekeeping gene, β-actin (dashed line), *CXCL10* had the lowest baseline expression across all genes of interest (**Figure 9A**). Despite considerable variability amongst dose-response curves, *CXCL10* was the most induced gene following IFN-γ treatment across all donors (**Figure 9A**). Moreover, *CXCL10* demonstrated significant induction [$P < 0.05$] even at the low dose of 0.1 U/mL of IFN-γ compared to other target genes, with seven out of ten donors demonstrating an above 100-fold increase in expression compared to no IFN-γ treatment (**Figure 9B**). Surprisingly, four out of ten donors demonstrated an over 1000-fold increase in *CXCL10* expression at the lowest treatment dose [$P < 0.05$] (**Figure 9B**). The highest induction of *CXCL10* at 0.1 U/mL IFN-γ was Donor 5 at 6200-fold change (**Figure 9B**). Peak *CXCL10* induction occurred at 10 U/mL IFN-γ for 4 out of 10 donors and at 100 U/mL IFN-γ for 6 out of 10 donors (**Figure 9B**).

CCL5 had the second lowest baseline expression compared to β-actin (**Figure 9A**). Unlike *CXCL10*, *CCL5* did not demonstrate a strong dose-response to IFN-γ and was the least induced out of all target genes (**Figure 9B**).

Even though *IL-15* and *IL-12* demonstrated similar baseline level expression, they varied in induction following IFN-γ treatment (**Figure 9A**). Compared to *CXCL10, IL-15* was the next most induced gene at all doses of IFN-γ and demonstrated a modest but weaker dose-response curve with peak gene induction at 10 U/mL IFN-γ in 9 out of 10 donors (**Figure 9B**). Following *IL-15*, *IL-12A* was the next most induced gene at all doses but demonstrated a less defined dose response to IFN-γ (**Figure 9B**). Peak *IL-12A* induction was observed at 10 U/mL in 4 out of 10 donors and

at 1.0 U/mL IFN-γ in 4 out of 10 donors; however, induction did not significantly increase following 0.1 U/mL IFN-γ across all donors (**Figure 9B**). Moreover, the lack of significant *IL-12A* induction at higher doses of IFN-γ suggests that lower doses of IFN-γ are sufficient for maximum *IL-12A* expression.

IFI16 demonstrated high baseline expression levels in all donors comparable to β-actin, which may contribute to its modest induction following IFN-γ treatment (**Figure 9A**). *IFI16* expression increased slightly following IFN-γ, falling just below a five-fold increase in 9 out of 10 donors, which was maintained regardless of IFN-γ dose (**Figure 9B**).

These observations reveal several essential insights. Keratinocytes exhibit high sensitivity to IFN-γ, corroborated by observable gene induction at even low doses of IFN-γ across all donors and all gene targets. Moreover, donor-derived keratinocytes display highly variable dose-response curves, suggesting donor-dependent responses. Despite variable induction, *CXCL10* is dramatically induced across all donors, even at the lowest dose of IFN-γ. This sensitive and robust response suggests that *CXCL10* plays a key role in IFN-γ-mediated inflammatory responses. Considering IFN-γ is enriched in HSV-affected skin during and after viral recurrence, we hypothesize that *CXCL10* expression is likewise dramatically induced *in vivo*. Other genes, such as *IFI16*, demonstrate little to no dose-dependent effect, suggesting alternative pathways and immune roles. Furthermore, this analysis reveals distinct IFN-γ dosages required for maximum gene induction. Maximum gene induction is observed in response to 1.0 to 10 U/mL IFN-γ in most donors. This observation provides insight into the optimal therapeutic dose necessary and sufficient for maximum target gene induction.

3.3 IFNγ-Induced Gene Expression is Time-Dependent in Keratinocytes

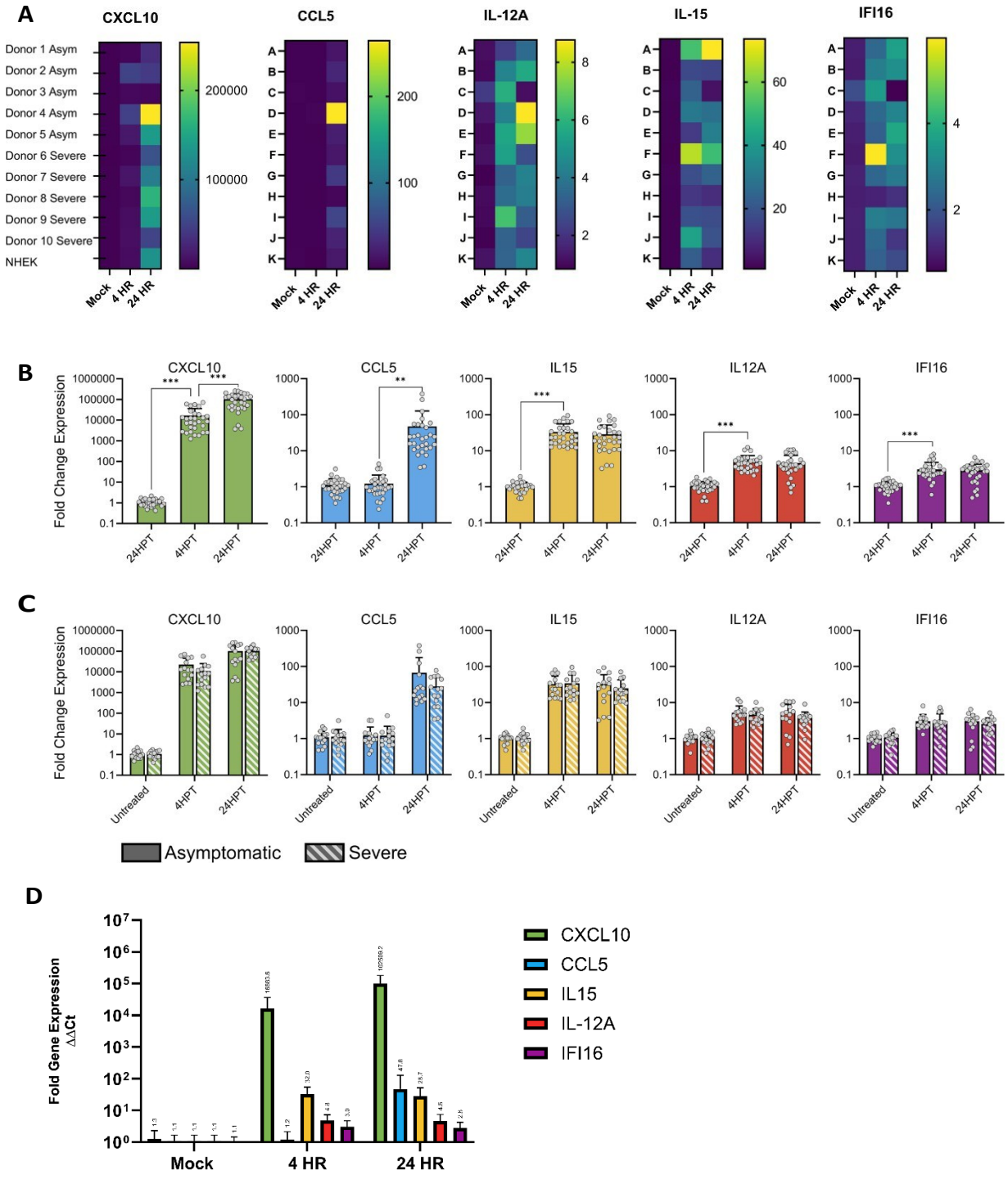

Figure 10 Expression of IFN-γ induced gene expression following different incubation incubations times with IFN-γ (A) Heat maps showing gene induction in donor-derived primary keratinocytes (n = 10) mock-treated or treated with 100 U/mL IFN-γ at 4 hours and 24 hours post-treatment. **(B)** Bar graph quantification of *CXCL10, CCL5, IL-15, IL-12A*, and *IFI16* with respect to incubation time (n=30). **(C)** Comparison of fold change in gene expression in asymptomatic versus severe HSV-2 donors. **(D)** Bar graph depicting quantitative gene induction in all donors (n = 10). Fold change was calculated with respect to mock expression normalized to β-actin.

To elucidate whether IFN-γ incubation time influences gene expression, we performed a transcriptional analysis of primary human keratinocytes following a 4-hour or 24-hour incubation time with 100 U/mL of IFN-γ. Given that all target genes demonstrated induction at 100 U/mL IFN-γ (**Figure 10**), we decided to treat primary keratinocytes with a higher dose of 100 U/mL IFN-γ to eliminate variability in gene expression due to IFN-γ dose.

Most donors demonstrated little to no baseline expression of *CXCL10*, *CCL5*, and *IL-15* (**Figure 10C**). All donors demonstrated detectable, low levels of *IFI16* expression prior to IFN-γ treatment, further strengthening the evidence that *IFI16* is constitutively expressed (**Figure 10A, Table 3**). After a 4-hour IFN-γ incubation period, all genes demonstrated significant induction, with the exception of *CCL5* (**Figure 10A, B, D**). *CXCL10* was induced up to 71320-fold at 4 hours and up to 257956-fold at 24 hours, suggesting continuous transcription of *CXCL10* or a potential positive feedback loop ($P < 0.001$). Interestingly, while we did not detect *CCL5* expression at 4 hours, *CCL5* was significantly induced at 24 hours in most donors (50-fold change, $P < 0.01$) (**Figures 10A, B, D**). *IL-12A* and *IL-15* expression was highly variable amongst donors at 4 and 24 hours. 7 out of 10 donors demonstrated increased *IL-12A* expression at 24 hours compared to 4 hours, while only 3 out of 10 donors demonstrated an increase in *IL-15* at 24 hours (**Figures 10A, B**). On average, however, no significant difference in fold change was detected between incubation times for *IL-12A* and *IL-15* (**Figure 10B).** *IFI16* expression was upregulated at 4 hours ($P < 0.001$) but was unchanged between the two incubation times (**Figure 10B**). Additionally, we observed no significant difference in gene induction at 4 or 24 hours amongst asymptomatic and severe donors at this dose (**Figure 10D**).

3.4 Keratinocytes Demonstrate Higher Interferon-Stimulated Gene Expression Compared to Other Structural Cells

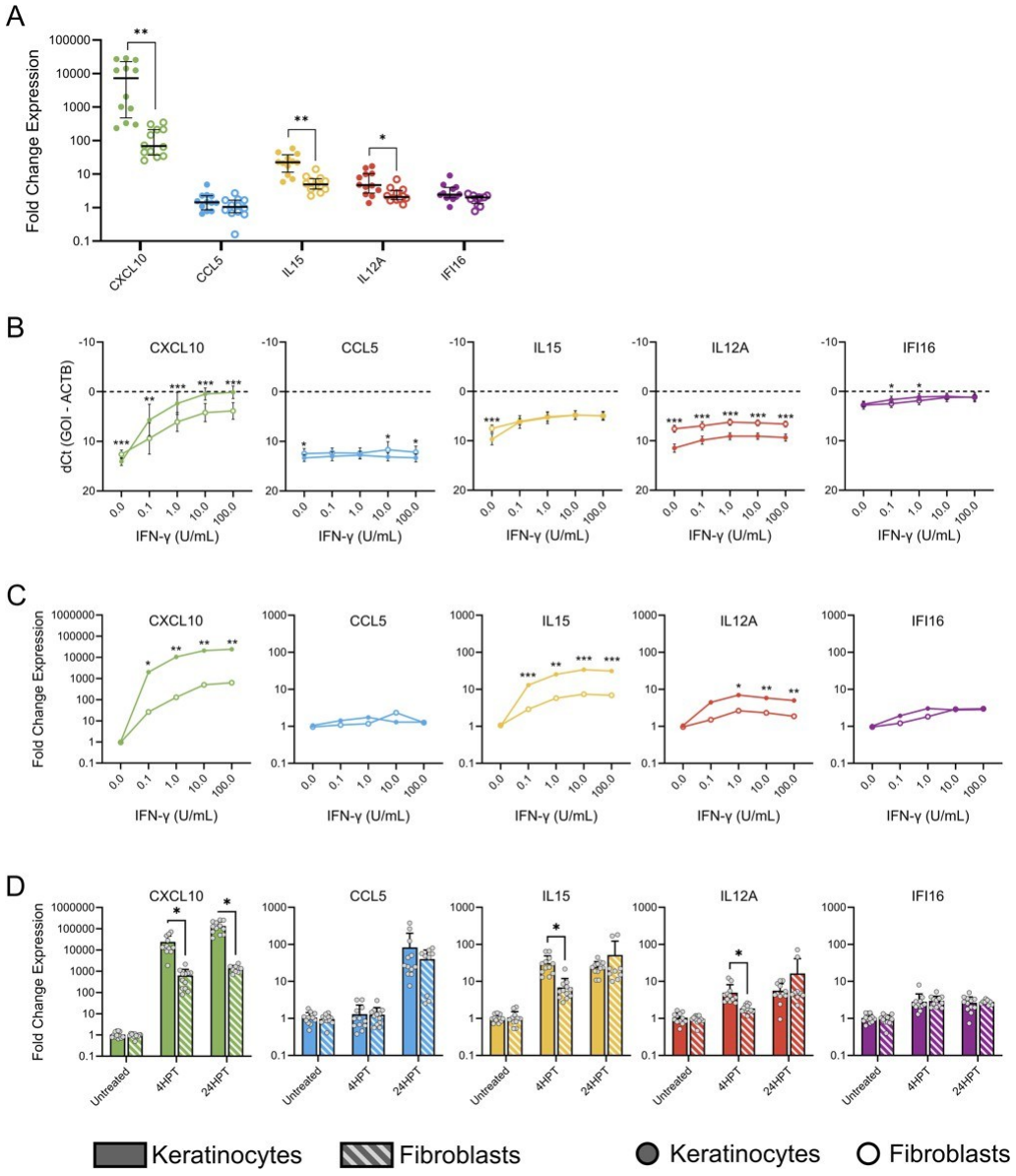

Figure 11 Comparison of IFN-γ induced gene expression in donor-derived keratinocytes (n = 12) and matched donor-derived fibroblasts (A) Representation of overall fold change in *CXCL10, CCL5, IL-15, IL-12A,* and *IFI16* expression in primary donor keratinocytes (n = 12) and matched-donor fibroblasts (n = 12) in response to 1.0 U/mL IFN-γ 4 h.p.t. **(B)** Induction of *CXCL10*, *CCL5, IL-15, IL-12A,* and *IFI16* expression in primary donor keratinocytes (n = 12) and donor-matched fibroblasts (n = 12) normalized to β-actin (dashed line). Data is represented as dCt, which is calculated by subtracting the Ct value of induced genes from β-actin Ct values. All Ct values were acquired directly from qPCR with a maximum Ct threshold of 35. **(C)** Fold change in *CXCL10*, *CCL5, IL-15, IL-12A,* and *IFI16* expression in primary donor keratinocytes and matched-donor fibroblasts 4 h.p.t.. Fold change calculated as relative to mock expression, normalized to β-actin. **(D)** IFN-y-induced gene expression at 4- and 24-hours post-treatment in keratinocytes and donor-matched fibroblasts.

While HSV primarily infects epidermal keratinocytes, fibroblasts, which reside deeper in the dermis, are susceptible to infection and play a role in cell-mediated immunity. To determine whether IFN-γ elicits cell-type-specific gene expression, we performed a dose-response analysis in donor-matched primary keratinocytes and fibroblasts to examine differential expression of *CXCL10, CCL5, IL-15, IL-12A*, and *IFI16*.

Overall, donor-derived primary keratinocytes demonstrated significantly higher induction of *CXCL10*, *IL-15,* and *IL-12A* than fibroblasts in response to 1.0 U/mL IFN-γ (**Figure 11A**). In particular, keratinocytes demonstrated an average 10450-fold change in *CXCL10* expression compared to the 130-fold change in *CXCL10* expression in fibroblasts ($P < 0.01$). Concerning *IL-15* induction, keratinocytes and fibroblasts exhibited an average 25- and 5.8-fold change in expression, respectively ($P < 0.01$). Lastly, keratinocytes and fibroblasts exhibited an average 7- and 2.6-fold change in IL-12A expression, respectively ($P < 0.05$).

To account for differences in baseline expression of target genes, we compared baseline expression of *CXCL10, CCL5, IL-15, IL-12A,* and *IFI16* in keratinocytes and fibroblasts. Our analysis revealed that keratinocytes exhibit higher **baseline expression** of *CXCL10* and *IL-15* ($P < 0.01$) as well as *CCL5* ($P < 0.05$) (**Figure 11B**). In contrast, we observed significantly higher *IL-12A* baseline expression in fibroblasts, which may account for less dramatic induction of these genes in response to IFN-γ ($P < 0.001$) (**Figure 11B**).

Our dose-response analysis revealed that keratinocytes exhibit higher *CXCL10* and *IL-15* expression in response to *every dose* of IFN-γ ($P < 0.05$ for 0.1 U/mL and $P < 0.01$ *for* IFN-γ doses above 0.1 U/mL) (**Figure 11C**). Notably, keratinocytes were more sensitive to lower doses of IFN-γ; expression of *CXCL10* in keratinocytes increased by approximately 2000-fold at 0.1 U/mL IFN-γ, while fibroblasts demonstrated a significantly lower 27-fold change in expression at the same dose (**Figure 11C**). Additionally, keratinocytes exhibited significantly higher *IL-12A* induction than fibroblasts at IFN-γ doses of 1.0 U/mL and above ($P < 0.05$ for 1.0 U/mL and $P < 0.01$ *for* IFN-γ doses above 1.0 U/mL) (**Figure 11C**).

Both cell types demonstrated a mild increase in *CCL5* and *IFI16* expression in response to IFN-γ; however, we observed no significant difference in IFN-γ dose response between keratinocytes and fibroblasts (**Figure 11C**).

To determine whether keratinocytes and fibroblasts exhibit differential gene expression in response to IFN-γ incubation time, we compared gene induction at 4 hours and 24 hours post-100 U/mL IFN-γ treatment. Both cell types demonstrated increased *CCL5* expression at 24 hours post-treatment with no significant difference in induction between

keratinocytes and fibroblasts (**Figure 11D**). Keratinocytes exhibited significantly higher *CXCL10* induction at both 4- and 24-hour post-IFN-γ treatment ($P < 0.05$) (**Figure 11D**). While keratinocytes demonstrated substantially higher induction of *IL-15* and *IL-12A* at 4 hours, we did not observe a significant difference in *IL-15* or *IL-12A* induction at 24 hours post-IFN-γ treatment (**Figure 11D**). *IFI16* expression did not increase with time in either cell type, suggesting no cell-type specificity (**Figure 11D**).

3.5 Dose Response Analysis Reveals Significant Difference in CXCL10 Induction in Asymptomatic versus Severe Individuals

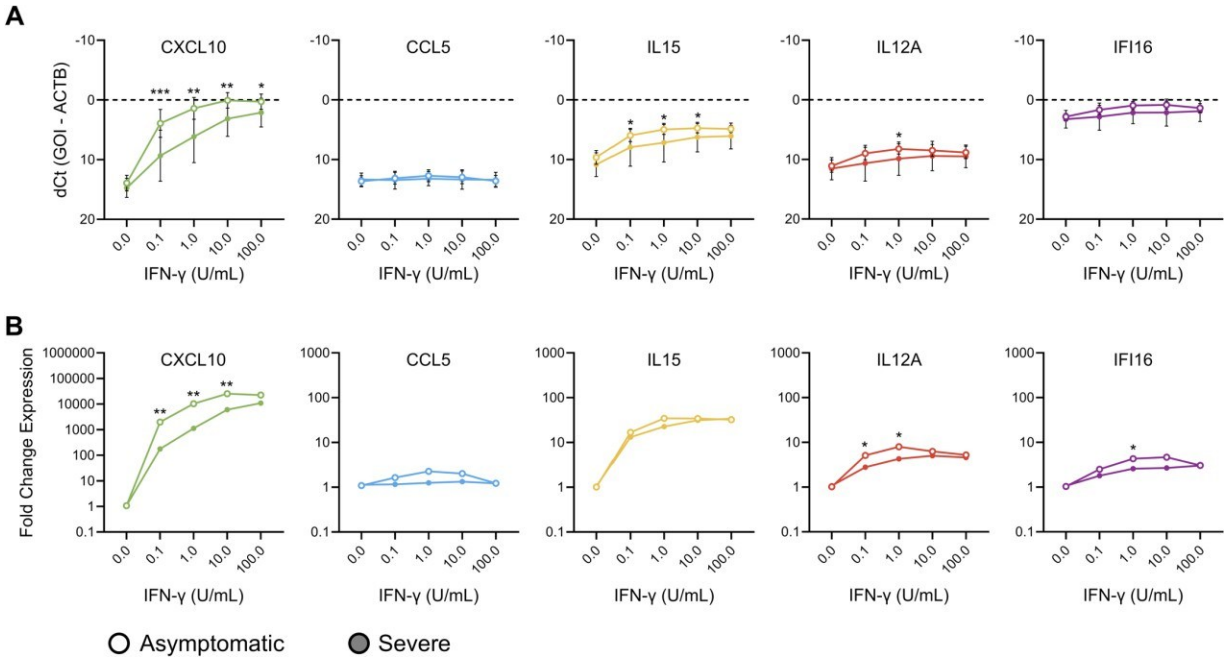

Figure 12 Comparison of gene induction in response to IFN-γ in asymptomatic versus severe donors (A) Comparison of *CXCL10*, *CCL5, IL-15, IL-12A,* and *IFI16* induction in response to different concentrations of IFN-γ (0.1 to 100 U/mL) in primary donor keratinocytes derived from asymptomatic (colored solid line) and severe HSV-2 donors (colored dashed line). Gene expression normalized to β-actin (dashed line). Data is represented as dCt, which is calculated by subtracting the Ct value of induced genes from β-actin Ct values. All Ct values were acquired directly from qPCR with a maximum Ct threshold of 35. **(B)** Fold change in *CXCL10*, *CCL5, IL-15, IL-12A,* and *IFI16* expression calculated as relative to mock expression normalized to β-actin.

To determine whether there is a correlation between HSV-2 disease severity and IFN-γ-induced gene expression, we analyzed the difference in average gene induction by separating our cohort based on whether they are asymptomatic or experience symptomatic, severe HSV-2 reactivations. In this study, having a severe phenotype is defined as experiencing more than 6 ulcers per year.

Our analysis revealed that while *CXCL10* displays the highest induction across all genes, keratinocytes derived from asymptomatic individuals demonstrate significantly higher *CXCL10* induction in response to 0.1, 1.0, and 10 U/mL IFN-γ (**Figure 12**). While there is no difference in baseline *CXCL10* expression amongst donors, *CXCL10* expression in keratinocytes acquired from asymptomatic donors increased by approximately 2000-fold at 0.1 U/mL IFN-γ; in contrast, keratinocytes from severe donors demonstrated a significantly lower 175-fold *CXCL10* induction (**Figures 12A, B**). Moreover, asymptomatic individuals continuously demonstrated significantly higher *CXCL10* induction at 1.0 and 10

U/mL IFN-γ ($P < 0.01$) (**Figure 12B**). 10 U/mL IFN-γ induced peak *CXCL10* expression in asymptomatic donors with an average 25000-fold change in expression. The equivalent dose induced only a 6000-fold change in severe donors ($P < 0.01$).

While there was no significant difference in *CCL5* and *IL-15* induction in response to IFN-γ amongst donors, the asymptomatic cohort exhibited slightly more expression at doses lower than 100 U/mL IFN-γ (**Figure 12B**). Interestingly, asymptomatic donors demonstrated significantly higher *IL-12A* induction in response to 0.1 and 1.0 U/mL IFN-γ despite no difference in baseline *IL-12A* expression (**Figures 12A, B**). Likewise, asymptomatic individuals demonstrated slightly higher IFI16 induction; however, there was no significant difference amongst cohorts (**Figure 12B**).

Despite a limited donor pool, a significantly higher and earlier *CXCL10* induction was observed in asymptomatic individuals at low doses of IFN-γ, suggesting a possible mechanism behind asymptomatic HSV reactivation. While asymptomatic individuals demonstrated higher gene induction in response to IFN-γ overall, a bigger sample size is necessary to extract significant differences between cohorts.

3.6 HSV-2 Dampens IFN-γ-Mediated Gene Induction of CXCL10

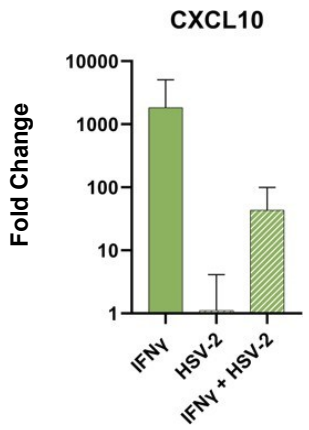

Figure 13 HSV-2 inhibits CXCL10 induction in primary keratinocytes Primary keratinocytes (n = 2) were either treated with 1.0 U/mL IFN-γ, infected with HSV-2 (MOI = 1) or pretreated with 1.0 U/mL IFN-γ and infected with HSV-2 (MOI = 1). All cells were collected 4 hours post treatment or post infection. Fold change in *CXCL10* expression calculated as relative to mock expression normalized to β-actin.

HSV-2 has been demonstrated to mediate cytokine and chemokine production as a countermeasure to evade host innate immune responses [90]. We wanted to assess how HSV-2 infection affects IFN-γ-induced expression of *CXCL10.* As expected, we observed a robust induction of *CXCL10* expression following treatment with 1.0 U/mL IFN-γ (**Figure 13**). HSV-2 infection alone did not induce significant *CXCL10* expression. Pretreatment with 1.0 U/mL IFN-γ followed by HSV-2 infection induced *CXCL10* expression, but at a much lower fold change than IFN-γ treatment alone (**Figure 13**). Our data indicate that HSV-2 successfully suppresses IFN-γ-mediated *CXC10* expression. We hypothesize that HSV-2 either targets *CXCL10* transcription directly or suppresses a pathway within IFN-γ signaling.

Chapter 4. Discussion and Limitations

Herpes simplex virus type 2 (HSV-2) causes lifelong recurrent genital ulcerative disease, affecting millions of people worldwide. Frequent viral reactivation manifests as symptomatic ulcers or subclinical, asymptomatic shedding episodes. Virological and immunological variations in the tissue microenvironment likely contribute to the wide range of clinical outcomes and disease manifestations observed at the individual level. Despite the effectiveness of antiviral interventions, an HSV vaccine remains lacking, underscoring the importance of understanding the immune responses required to control HSV infection in humans.

Rapid clearance and early containment of HSV-2 emphasize the importance of a targeted, localized immune response. A unique subset of CD8+ T cells, termed tissue-resident memory T cells (CD8+ T_{RM}), has been demonstrated to be HSV-2-specific and persist at the sites of prior infection [20]. Through rapid recognition and elimination of infected cells, tissue-resident memory T cells provide on-site and rapid protection against chronic and recurrent HSV-2 infection [20, 14].

Despite their persistence and potent cytotoxic activities, the underlying mechanism behind effective containment and tissue-wide protection by a handful of resident T cells remains unknown. Studies in human genital HSV-2 infection revealed robust immunological crosstalk between CD8+ T_{RM} and their neighboring epithelial cells [20]. CD8+ T_{RM} production of IFN-γ and subsequent signaling instructs broad innate and cell-intrinsic antiviral defense in neighboring keratinocytes [20]. Notably, IFN-γ induces the expression of a broad spectrum of restriction factors, chemoattractants, and antiviral signaling pathways that effectively block HSV-2 invasion and promote tissue-wide protection.

A detailed characterization of the magnitude, function, and specificity of IFN-γ-mediated immune signaling can help elucidate the mechanism behind effective viral containment. Additionally, differential expression of IFN-γ-mediated signaling can reveal a potential source of variability in disease severity in HSV-2-affected individuals. Access to HSV-2-positive, donor-derived primary cell lines establishes an invaluable platform for examining donor-specific immune responses, further providing a natural segue into biologically relevant modeling systems such as organoids and skin-on-chip platforms. A thorough and targeted immune response analysis (i.e., how a subset of ISGs respond to IFN-γ and control asymptomatic HSV-2 reactivation) is essential to elucidate the optimal immune response to HSV-2 and design a vaccine strategy that can emulate this level of local protection.

Based on a thorough transcriptional analysis of a subset of IFN-γ-stimulated genes, our studies revealed distinct gene expression patterns in donor-derived primary cell lines that provide essential insights into the function, magnitude, and differential response to immune signaling during HSV-2 infection.

4.1 Insights into Donor Variation

Overall, donor-derived primary keratinocytes demonstrated considerable variability in gene expression following IFN-γ stimulation [Figure 8]. We suspect these differences in expression and functional regulation of IFN-γ-stimulated genes contribute to the broad spectrum of clinical outcomes observed in human genital herpes infection.

Despite individual variation, IFN-γ elicited distinct patterns of *CXCL10, CCL5, IL-15, IL-12A*, and *IFI16* induction. *CXCL10* was robustly induced, experiencing up to 60956 maximum fold change in expression following treatment with 0.1 U/mL IFN-γ [Figure 8]. In fact, *CXCL10* consistently demonstrated maximum induction across all of our studies. Other genes, such as *CCL5* and *IFI16*, demonstrated significantly lower induction in response to the same dose of IFN-γ. A widely accepted hypothesis in the field of immunology proposes that the genes most highly induced during infection or IFN treatment control viral replication most effectively. Thus, we suspect that the overexpression of *CXCL10* reflects its salient role in early immune defense.

Regarding moderately induced genes, we presume that alternative signaling pathways contribute to their limited response to IFN-γ. Moreover, some genes might be biologically active at lower concentrations or play a minor role in IFN-γ-mediated antiviral defense in keratinocytes. Establishing the average induction and range of expression for differentially expressed genes increases our understanding of the magnitude of induction in response to IFN-γ in keratinocytes, allowing us to predict the magnitude of induction of a specific gene following IFN treatment in future studies. This model allowed us to capture strong upregulation of IFN-γ-stimulated genes in uninfected keratinocytes, contributing to data that emphasizes the role of cytokines in instigating a potent alarm response.

4.2 Dose Response Analysis

Our data indicate that keratinocytes are extremely sensitive to IFN-γ and efficiently respond to cytokine signaling. Target genes were significantly induced after minimal IFN-γ stimulation. 0.1 U/mL of IFN-γ was sufficient to stimulate significant induction of antiviral genes in about 100,000 nearby keratinocytes. We suspect this heightened sensitivity contributes to the rapid and potent antiviral response observed in vivo.

Maximum change in gene expression of ISGs occurred after 1.0 or 10.0 U/mL IFN-γ treatment, highlighting IFN-γ's ability to elicit a potent response at significantly lower concentrations than previously reported. Most prior studies involving IFN-γ utilize up to 1000 U/mL to induce expression, which could lead to both artificial overexpression and a

lack of detection of peak induction. Here, we demonstrate that a dose of 10.0 U/mL or below elicits just as strong, if not maximum, expression of IFN-γ target genes [Figure 9].

Moreover, our dose-response analysis revealed a dramatic upregulation of *CXCL10* following minimal IFN-γ treatment [Figure 9]. Treatment with 0.1 U/mL IFN-γ resulted in over a 1000-fold change in *CXCL10* expression and up to 16,000-fold in response to 10.0 U/mL IFN-γ [Figure 9]. This dramatic induction confirms our hypothesis that *CXCL10* is a significant target of IFN-γ signaling and might play a vital role in the initial antiviral immune response. This aligns with *CXCL10*'s function as a chemoattractant. Chemoattractants are rapidly induced in response to viral infection and trigger extensive signaling cascades in target cells [97]. The faster and stronger the chemotactic gradient, the greater the mobilization of immune cells to sites of infection, trauma, or inflammation [97]. Exceptionally high *CXCL10* transcription suggests that *CXCL10* is an essential and potent chemoattractant whose upregulation likely results in a powerful gradient, increased recruitment of immune cells, and faster control of HSV-2 reactivation. Organoid models could help examine the extent of this chemotactic gradient and how it affects the spatiotemporal organization and mobilization of key cell types implicit in antiviral defense, including T cells, NK cells, and neutrophils. Further examination of the effect of IFN-γ dose in alternative models is necessary to understand how a biologically complex environment affects sensitivity. Ultimately, our dose-response analysis begins to unveil how a limited amount of CD8+ T_{RM}s produces enough IFN-γ to stimulate nearby epithelial cells and confer tissue-wide protection during HSV-2 reinfection.

4.3 Time-dependent Gene Induction

The timing of an antiviral response is a critical determinant of the extent of viral replication and subsequent pathology. The more rapidly a pathogen can be contained, the less pathology it or the immune response it provokes can cause. We demonstrate that IFN-γ can elicit a potent and timely response in keratinocytes. Moreover, a subset of genes exhibited time-dependent induction that revealed unique insights into their regulation and function. Notably, *CXCL10* demonstrated significant induction at 4 hours post-IFN-γ treatment, which significantly increased at 24 hours post-treatment. This data suggests that *CXCL10* is likely continuously transcribed and is implicated in long-term control. Considering *CXCL10* exhibited minimal baseline expression prior to stimulation, it's likely that *CXCL10* is strictly regulated despite its potent increase in transcription. Further investigation of *CXCL10* transcriptional regulation and in vivo activity would provide additional insight into its role in immune signaling.

In addition to *CXCL10, IL-15, IL-12A*, and *IFI16* demonstrated significant induction at earlier timepoints. Unlike *CXCL10*, we observed no significant increase in expression at later timepoints, suggesting that minimal IFN-γ stimulation is sufficient for peak *IL-15, IL-12A*, and *IFI16* expression. Importantly, expression of *IL-15, IL-12A*, and *IFI16* was maintained for at least 24 hours, highlighting the long-lasting effect of IFN-γ. This aligns with studies that demonstrate heightened, persistent levels of antiviral gene expression in post-healed skin [20]. Prolonged expression may reflect the role of *IL-15* and *IL-12A* in memory T cell maintenance and retention following HSV-2 infection. Continuous expression of *IFI16* 24 hours after stimulation likewise suggests a role in viral surveillance.

CCL5, also known as RANTES, is another critical chemokine primarily involved in the recruitment of natural killer (NK) cells during HSV-2 infection. Interestingly, while *CCL5* failed to induce at an earlier time point, we observed significant induction 24 hours post-IFN-γ treatment. We hypothesize that transcription of *CCL5* might require additional stimuli in addition to IFN-γ. Alternatively, delayed *CCL5* induction suggests a more prominent role in later T cell maintenance and memory formation as opposed to acute chemotactic activity. Additionally, *CCL5* is known to be expressed mainly in macrophages in response to HSV-2, which might explain its limited induction in keratinocytes [68]. Moving forward, it would be interesting to inspect whether there is a difference in the temporal regulation of *CCL5* among cell types.

Our data indicate that specific IFN-γ-stimulated genes are under temporal regulation. Differences in induction in response to IFN-γ incubation time can help elucidate which target genes play a critical role in the acute immune response to HSV-2 and which genes can be evaluated for optimizing T cell maintenance.

4.4 Disease Severity

To elucidate whether HSV-2 disease severity correlates with IFN-γ-induced gene expression, we analyzed the difference in average gene induction in asymptomatic versus severe donors following IFN-γ stimulation. Our analysis revealed that primary keratinocytes from asymptomatic individuals are more sensitive to IFN-γ stimulation. While both cohorts demonstrated no difference in baseline expression of *CXCL10, CCL5, IL-15, IL-12A*, and *IFI16*, asymptomatic donors exhibited significantly higher induction of *CXCL10, IL-12A*, and *IFI16* following IFN-γ treatment [Figure 12]. Moreover, we observed the most significant difference in induction at low IFN-γ concentrations. Notably, asymptomatic donors exhibited the greatest fold change in *CXCL10* expression following minimal IFN-γ stimulation, which was maintained with increasing IFN-γ concentrations.

Similarly, the induction gap in *IL-12A* and *IFI16* between cohorts was most apparent at 1.0 and 10 U/mL IFN-γ treatments. This disparity decreased with higher concentrations of IFN-γ, suggesting that a sufficiently concentrated signal can override differences in sensitivity to IFN-γ. Nonetheless, asymptomatic donors not only responded more robustly to lower concentrations but also demonstrated higher gene induction at every dose of IFN-γ. Together, our data indicate that keratinocytes from asymptomatic donors are poised to respond earlier and more robustly to lower concentrations of IFN-γ.

This phenotype likely contributes to the immediate and robust antiviral response characteristic of asymptomatic HSV-2 reactivation. Moreover, the mechanism behind increased sensitivity to IFN-γ stimulation is of great interest to us; understanding how it contributes to the asymptomatic resolution of chronic infections can help us optimize the host immune response.

One potential explanation for increased sensitivity in keratinocytes is prior exposure to this cytokine or increased immune signaling. Multiple studies demonstrated that rapid HSV-2 containment can be attributed to a higher quantity of tissue-resident memory T cells at the site of reactivation [20]. By this reasoning, an increased CD8+ T_{RM} volume represents a larger source of IFN-γ, which can then stimulate more keratinocytes. A potential analysis we could perform would involve comparing TRM counts among donors with our current gene induction data. Additionally, we plan to acquire and stimulate donor-specific T cells to determine the magnitude of cytokine production. This would allow us to confirm biologically relevant IFN-γ concentrations and control for variation in IFN-γ production among donors.

Individual variation in the recognition and response to infection can also be attributed to differences in signaling transduction pathways, which have a genetic basis [98, 99]. Thus, we suspect that differential gene expression may be due to differences in upstream signaling or, more broadly, variation in genetic background. To confirm that donor variation is not due to differences in IFN-γ signaling, we investigated potential variation in *IFNGR1*, *STAT1*, and *STAT3* expression via RT-PCR. Our data indicated that there is no significant variation among our donors. Alternatively, we have begun to analyze differences in *STAT1*/*STAT3* localization in response to varying IFN-γ concentrations at different time points. So far, our data confirm that phosphorylated *STAT1*/*STAT3* successfully and swiftly migrate to the nucleus following IFN-γ stimulation. We have yet to determine whether there is significant variation in STAT localization speed and quantity.

In addition to IFN-γ signaling, we hypothesize that variation in gene expression is due to differences in transcriptional regulation of target genes. Differences in promoter activity and accessibility, as well as the quantity and binding efficiency of transcription factors, can result in the differential expression of genes. One way we plan to assess promoter activity is to use ChIP-seq, which would allow us to analyze the interactions of various transcription factors and other proteins with specific DNA segments in our donor-derived cells. A higher binding affinity would indicate a potential mechanism behind the increased or continuous transcription of genes, such as *CXCL10*. Additionally, it would be interesting to observe how specific genes, such as *IFI16*, are constitutively expressed and how genes with little to no baseline expression can be induced up to 1000-fold in as little as 4 hours (such as the case with *CXCL10*).

Classifying immune responses by age or sex might reveal additional insights into variable immune reactivity. Most evidence suggests that flexibility in immune responses becomes constrained with age [99]. For this analysis, we would require a significantly larger cohort. Alternatively, we could perform a retrospective study comparing disease severity with viral shedding rates, viral genome copies, immune cell counts, prior history of exposure to viral infections, age, and sex to evaluate the contribution of each variable to HSV-2 disease manifestation.

4.5 IFN-γ Cell Bias

While basal keratinocytes are the primary target of recurrent HSV-2 infection, HSV-2 can productively infect other cell types, including dermal fibroblasts, endothelial cells, neurons, and immune cells. Multiple studies have utilized fibroblasts to demonstrate HSV-2 infection and the response to IFN-γ stimulation; however, the sensitivity and response of fibroblasts to cytokine signaling have not been fully described [25, 37].

Our data indicate that keratinocytes are more sensitive to IFN-γ signaling than fibroblasts. Primary keratinocytes express significantly more *CXCL10, IL-15*, and *IL-12A* in response to IFN-γ than donor-matched fibroblasts, with no difference in the induction of *IFI16* and *CCL5* [Figure 11]. We observed consistently high baseline expression of *IFI16* across donor-derived keratinocytes and fibroblasts, suggesting that *IFI16* is an important cell-intrinsic defense mechanism in multiple cell types and is constitutively expressed in the epithelium. Prior studies have highlighted the increased expression of *IFI16* in HSV-2-affected epidermis, which is inversely correlated with viral genome copies [20]. Our data indicate that *IFI16* expression is not limited to HSV-affected skin, suggesting that *IFI16*-mediated antiviral defense extends to multiple anatomically distinct areas. It is of great interest whether *IFI16* confers resistance to other viruses and whether they share the capacity to target *IFI16* for degradation.

Furthermore, fibroblasts express significantly higher baseline *IL-12A* and *IL-15* than keratinocytes, which may account for their less dramatic induction following IFN-γ stimulation [Figure 11]. Constitutive, low-level expression of *IL-12A* and *IL-15* suggests that fibroblasts play a larger role in supporting immune cell responses and local immune memory. In contrast, keratinocytes are responsible for acute chemokine production and rapid antiviral defense. We hypothesize that this delineation is likely a product of spatioanatomical dynamics. As keratinocytes get infected, their ISG responses are geared towards the acute production of alarm signals for immune cell recruitment. Dermal fibroblasts reside deeper in the epidermis. This anatomic position may provide them with a spatial advantage to activate the proliferation and promote the maintenance of immune cells as they migrate toward the site of infection. Moreover, our data aligns with studies demonstrating constitutive expression of *IL-12A* and *IL-15* in fibroblasts near areas of prior HSV-2 infection. Significantly low baseline expression of chemokines such as *CXCL10*, which drastically increases after stimulation, suggests a tight regulation of cytokine expression that can help avoid unnecessary inflammation at the barrier surface.

4.6 HSV-2 Infection

To investigate the effect of viral infection on IFN-γ-mediated gene expression, we analyzed *CXCL10* induction after minimal IFN-γ stimulation and HSV-2 infection of donor-derived keratinocytes [Figure 13]. HSV-2 successfully dampened IFN-γ-mediated expression of *CXCL10*, confirming an evasion strategy of HSV to disarm host innate antiviral responses. Whether HSV-2 directly interacts with *CXCL10* transcription or interferes with the IFN-γ signaling pathway is of great interest. Prior studies have highlighted several viral proteins implicated in IFN signaling inhibition, including ICP0, ICP27, ICP34.5, and vhs [41]. However, we postulate that stronger IFN-γ stimulation may counteract HSV inhibitory effects. Determining a threshold that optimizes antiviral gene expression without excessive IFN-γ application would be beneficial for vaccine considerations.

Additionally, we are interested in analyzing the effect of HSV-2 infection on keratinocytes derived from both asymptomatic and severely affected individuals. Our data have indicated that *CXCL10* expression is significantly heightened in asymptomatic individuals. Whether this difference confers an immune advantage to viral infection is to be determined. We hypothesize that a stronger and faster upregulation of *CXCL10* could successfully overcome HSV-2-mediated inhibition. Observing the long-term effects of heightened *CXCL10* expression in a 3D model would elucidate the effect of *CXCL10* on cell migration and viral inhibition. Likewise, an infectious model system that demonstrates the impact of our findings would be optimal for vaccine and therapeutic development.

4.7 Limitations

We acknowledge that our analysis of donor-specific responses to immune signaling is limited in various ways. Our current sample size consists of 10 individuals, which limits the scope of our findings. However, the study size is comparable to that used in clinical trials, and our cohort represents a broad spectrum of clinical outcomes. Access to biopsy tissue from HSV-2 seropositive adults allowed us to extract primary cell lines and perform detailed characterization of their IFN-γ-mediated gene expression. Primary cells closely mimic an *in vivo* environment, maintaining natural morphology, metabolism, and responses to stimuli, and are ideal candidates for studying donor-specific responses. To account for a limited donor cohort, we selected individuals who were either asymptomatic or experienced severe HSV-2 reactivations. We excluded donors who experienced mild HSV-2 reactivations to minimize inconclusive results. However, we plan to corroborate our findings using HSV-2 shedding and viral titer data from all available donors. Additionally, we hope to acquire data from additional donors, perform a more extensive transcriptional analysis of all differentially expressed genes, and ultimately adopt a 3D organoid platform to model individual, multi-faceted immune responses.

Another limitation of our study involves the source of the tissue biopsies. Punch biopsies were acquired from the arm of HSV-2 seropositive, healthy adults. As such, these tissues were not directly exposed to HSV-2 infection and do not demonstrate gene expression profiles expected from HSV-2-affected tissue. HSV-2 can have long-term epigenetic effects on tissues, which can influence gene expression patterns and impact cellular processes [22, 85]. While our biopsy tissues may not represent an HSV-2-specific repertoire, the cells we derive from the arm are more representative of individual donor baseline gene expression of IFN-γ target genes in the absence of viral infection. Additionally, differences in epithelial cell sensitivities could be more representative of donor-specific genetic variation rather than a by-product of recurrent chronic disease. Moreover, we can assess the effect of HSV-2 on IFN-γ-mediated signaling in cells that were not predisposed to the virus to determine how HSV-2 epigenetically modifies cellular processes.

We further acknowledge the limitations of using a 2D monolayer model to investigate the effect of IFN-γ signaling. In vivo, cellular processes are exposed to a milieu of stimuli. The use of a homogenous cell culture is not entirely representative of the natural environment, including signaling, cellular cross-talk, and environmental cues that manipulate and shape intrinsic cellular responses. However, this model is sufficient for investigating specific cellular responses to a singular cytokine; otherwise, it would be challenging to discern independent effects from confounding variables. Thus, it was essential to establish an isolated system before incorporating additional cell types and

investigating multiple signaling pathways. We are interested in translating these findings and queries into a more complex 3D model that can provide a physiologically accurate environment for investigating immune mechanisms. For example, we intend to adapt the skin-on-chip system to incorporate key elements of HSV-2 disease, including intact skin architecture, functional endothelial vessels, infectious pathogens, immune components, and therapeutic agents. While 2D cultures offer indispensable insight into cellular processes and are commonly used to mimic in vivo conditions, we plan to implement 3D organoid models to represent the complexity of individual immune processes during HSV disease.

We must also acknowledge that our analysis involves a limited subset of IFN-γ-stimulated genes. These genes have been demonstrated to be induced by IFN-γ and implicated in HSV-2 recurrent disease. While prior studies have effectively identified differentially induced genes in response to infection and IFN-γ treatment, they do not provide a comprehensive analysis of the range and function of gene-specific induction. Moreover, our target gene subset encompasses a wide range of antiviral functions, including lymphocyte recruitment and activation, T cell retention and memory formation, as well as intrinsic cellular defense. Working with a limited but distinct set of target genes allows us to thoroughly analyze the regulation, function, and magnitude of their response. Ultimately, we aim to extend this study to other differentially expressed genes, providing a comprehensive analysis of cytokine and chemokine signaling in response to various stimuli. We are currently employing RNA sequencing to evaluate both gene expression and spatial dynamics of cytokine and chemokine signaling in donor biopsies. Similarly, we have expanded our study of CXCL10 to include related CXC compounds, such as *CXCL9* and *CXCL11*.

4.8 Concluding Remarks

HSV-2 infection and recurrent disease continue to be a public and global health concern. Elucidating the mechanism behind an effective host immune response can help us understand the determinants of disease severity and develop therapeutic strategies to contain and eradicate HSV-2. Differences in expression level and functional regulation of IFN-γ signature genes might contribute to the broad spectrum of clinical outcomes observed in HSV-2 infection. Here, we confirm that donor-derived structural cells demonstrate variable sensitivity to IFN-γ, which correlates with disease severity. Further investigation of the effect of differential ISG expression in HSV-2 infection is necessary to determine the mechanism behind an optimal host immune response. Adopting a multicellular, biologically relevant system would allow us to measure the effect of cytokine signaling and subsequent antiviral gene expression on immune cell activity and viral clearance. Ultimately, a thorough analysis of the local immune environment in a biologically complex system can allow us to modulate the effects of selected chemokines on immune phenotype and protection against HSV-2.

References

[1] Roizman B., Knipe D., Whitley R. Herpes Simplex Viruses. In: Knipe D.M., Howley P., editors. Fields Virology. 6th ed. Wolters Kluwer; Alphen aan den Rijn, The Netherlands: 2021.

[2] Smith JB, Herbert JJ, Truong NR, Cunningham AL. Cytokines and chemokines: The vital role they play in herpes simplex virus mucosal immunology. Front Immunol. 2022 Sep 23;13:936235. doi: 10.3389/fimmu.2022.936235. PMID: 36211447; PMCID: PMC9538770

[3] Mikloska Z, Danis VA, Adams S, Lloyd AR, Adrian DL, Cunningham AL. In vivo production of cytokines and beta (C-C) chemokines in human recurrent herpes simplex lesions--do herpes simplex virus-infected keratinocytes contribute to their production? J Infect Dis. 1998 Apr;177(4):827-38. doi: 10.1086/515236. PMID: 9534953.

[4] Roychoudhury P, Swan DA, Duke E, Corey L, Zhu J, Davé V, Spuhler LR, Lund JM, Prlic M, Schiffer JT. Tissue-resident T cell-derived cytokines eliminate herpes simplex virus-2-infected cells. J Clin Invest. 2020 Jun 1;130(6):2903-2919. doi: 10.1172/JCI132583. PMID: 32125285; PMCID: PMC7260013.

[5] Xiaoyu Hu, Lionel B. Ivashkiv, Cross-regulation of Signaling Pathways by Interferon-γ: Implications for Immune Responses and Autoimmune Diseases, Immunity, Volume 31, Issue 4, 2009, Pages 539-550, ISSN 1074-7613, https://doi.org/10.1016/j.immuni.2009.09.002.

[6] C. Johnston, DM Koelle, A. Wald Current status and prospects for development of an HSV vaccine *Vaccine*, 32 (2014), pp. 1553-1560

[7] McQuillan G, Kruszon-Moran D, Flagg EW, Paulose-Ram R. Prevalence of herpes simplex virus type 1 and type 2 in persons aged 14–49: United States, 2015–2016. NCHS Data Brief, no 304. Hyattsville, MD: National Center for Health Statistics. 2018.

[8] Looker KJ, Elmes JAR, Gottlieb SL, Schiffer JT, Vickerman P, Turner KME, Boily MC. Effect of HSV-2 infection on subsequent HIV acquisition: an updated systematic review and meta-analysis. Lancet Infect Dis. 2017 Dec;17(12):1303-1316. doi: 10.1016/S1473-3099(17)30405-X. Epub 2017 Aug 23. PMID: 28843576; PMCID: PMC5700807.

[9] Zhu S, Viejo-Borbolla A. Pathogenesis and virulence of herpes simplex virus. Virulence. 2021 Dec;12(1):2670-2702. doi: 10.1080/21505594.2021.1982373. PMID: 34676800; PMCID: PMC8923070.

[10] Phipps W, Saracino M, Magaret A, Selke S, Remington M, Huang ML, Warren T, Casper C, Corey L, Wald A. Persistent genital herpes simplex virus-2 shedding years following the first clinical episode. J Infect Dis. 2011 Jan 15;203(2):180-7. doi: 10.1093/infdis/jiq035. Epub 2010 Dec 9. PMID: 21288817; PMCID: PMC3058740

[11] McQuillan G, Kruszon-Moran D, Flagg EW, Paulose-Ram R. Prevalence of Herpes Simplex Virus Type 1 and Type 2 in Persons Aged 14-49: United States, 2015-2016. NCHS Data Brief. 2018 Feb;(304):1-8. PMID: 29442994.

[12] Looker KJ, Magaret AS, May MT, Turner KM, Vickerman P, Gottlieb SL, Newman LM. Global and Regional Estimates of Prevalent and Incident Herpes Simplex Virus Type 1 Infections in 2012. PLoS One. 2015 Oct 28;10(10):e0140765. doi: 10.1371/journal.pone.0140765. PMID: 26510007; PMCID: PMC4624804.

[13] Schiffer, J., Corey, L. Rapid host immune response and viral dynamics in herpes simplex virus-2 infection. *Nat Med* 19, 280–288 (2013). https://doi.org/10.1038/nm.3103

[14] Zhu J, Koelle DM, Cao J, Vazquez J, Huang ML, Hladik F, Wald A, Corey L. Virus-specific CD8+ T cells accumulate near sensory nerve endings in genital skin during subclinical HSV-2 reactivation. J Exp Med. 2007 Mar 19;204(3):595-603. doi: 10.1084/jem.20061792. Epub 2007 Feb 26. PMID: 17325200; PMCID: PMC2137910.

[15] Freeman EE, Weiss HA, Glynn JR, Cross PL, Whitworth JA, Hayes RJ. Herpes simplex virus 2 infection increases HIV acquisition in men and women: systematic review and meta-analysis of longitudinal studies. AIDS. 2006 Jan 2;20(1):73-83. doi: 10.1097/01.aids.0000198081.09337.a7. PMID: 16327322.

[16] Schiffer JT, Abu-Raddad L, Mark KE, Zhu J, Selke S, Koelle DM, et al. Mucosal host immune response predicts the severity and duration of herpes simplex virus-2 genital tract shedding episodes. Proc Natl Acad Sci U S A 2010 Oct 18; 107(44):18973-8.

[17] Schiffer JT, Abu-Raddad L, Mark KE, Zhu J, Selke S, Magaret A, et al. Frequent release of low amounts of herpes simplex virus from neurons: results of a mathematical model. *Sci Transl Med* 2009 Nov 18; 1(7):7ra16. doi:10.1126/scitranslmed.3000193. Pubmed PMID: 20161655

[18] Zhu J, Hladik F, Woodward A, Klock A, Peng T, Johnston C, Remington M, Magaret A, Koelle DM, Wald A, Corey L. Persistence of HIV-1 receptor-positive cells after HSV-2 reactivation is a potential mechanism for increased HIV-1 acquisition. Nat Med. 2009 Aug;15(8):886-92. doi: 10.1038/nm.2006. Epub 2009 Aug 2. PMID: 19648930; PMCID: PMC2723183

[19] Borase, H.; Shukla, D. The Interplay of Genital Herpes with Cellular Processes: A Pathogenesis and Therapeutic Perspective. *Viruses* 2023, *15*, 2195. https://doi.org/10.3390/v15112195

[20] Peng T, Phasouk K, Sodroski CN, Sun S, Hwangbo Y, Layton ED, Jin L, Klock A, Diem K, Magaret AS, Jing L, Laing K, Li A, Huang ML, Mertens M, Johnston C, Jerome KR, Koelle DM, Wald A, Knipe DM, Corey L, Zhu J. Tissue-Resident-Memory $CD8^+$ T Cells Bridge Innate Immune Responses in Neighboring Epithelial Cells to Control Human Genital Herpes. Front Immunol. 2021 Sep 6;12:735643. doi: 10.3389/fimmu.2021.735643. PMID: 34552595; PMCID: PMC8450389.

[21] Wakim LM, Waithman J, van Rooijen N, Heath WR, Carbone FR. Dendritic cell-induced memory T cell activation in nonlymphoid tissues. Science. 2008 Jan 11;319(5860):198-202. doi: 10.1126/science.1151869. PMID: 18187654.

[22] Sanchez EL, Lagunoff M. Viral activation of cellular metabolism. Virology. 2015 May;479-480:609-18. doi: 10.1016/j.virol.2015.02.038. Epub 2015 Mar 23. PMID: 25812764; PMCID: PMC4424078.[23] Berger A. Th1 and Th2 responses: what are they? BMJ. 2000 Aug 12;321(7258):424. doi: 10.1136/bmj.321.7258.424. PMID: 10938051; PMCID: PMC27457.

[24] Hu, X., Herrero, C., Li, WP. *et al.* Sensitization of IFN-γ Jak-STAT signaling during macrophage activation. *Nat Immunol* 3, 859–866 (2002). https://doi.org/10.1038/ni828

[25] Koelle DM, Tigges MA, Burke RL, Symington FW, Riddell SR, Abbo H, Corey L. Herpes simplex virus infection of human fibroblasts and keratinocytes inhibits recognition by cloned CD8+ cytotoxic T lymphocytes. J Clin Invest. 1993 Mar;91(3):961-8. doi: 10.1172/JCI116317. PMID: 8383706; PMCID: PMC288048.

[26] Zhu J, Miner MD. Local Power: The Role of Tissue-Resident Immunity in Human Genital Herpes Simplex Virus Reactivation. Viruses. 2024 Jun 25;16(7):1019. doi: 10.3390/v16071019. PMID: 39066181; PMCID: PMC11281577.

[27] Leib, D.A. Counteraction of interferon-induced antiviral responses by herpes simplex viruses. *Curr. Top. Microbiol. Immunol.* 2002, *269*, 171–185.

[28] Katze, M., He, Y. & Gale, M. Viruses and interferon: a fight for supremacy. Nat Rev Immunol 2, 675–687 (2002). https://doi.org/10.1038/nri888

[29] Boehm U, Klamp T, Groot M, Howard JC. Cellular responses to interferon-gamma. Annu Rev Immunol. 1997;15:749-95. doi: 10.1146/annurev.immunol.15.1.749. PMID: 9143706.

[30] Schiffer JT, Swan D, Al Sallaq R, Magaret A, Johnston C, Mark KE, et al. Rapid localized spread and immunologic containment define Herpes simplex virus-2 reactivation in the human genital tract. *Elife* 2013 Apr 16; 2:e00288. doi:10.7554/eLife.00288. Pubmed PMID: 23606943

[31] Bhat MY, Solanki HS, Advani J, Khan AA, Keshava Prasad TS, Gowda H, Thiyagarajan S, Chatterjee A. Comprehensive network map of interferon gamma signaling. J Cell Commun Signal. 2018 Dec;12(4):745-751. doi: 10.1007/s12079-018-0486-y. Epub 2018 Sep 6. PMID: 30191398; PMCID: PMC6235777.

[32] Liu M, Guo S and Stiles JK: The emerging role of CXCL10 in cancer (Review). Oncol Lett 2: 583-589, 2011.

[33] Moser, B., Loetscher, P. Lymphocyte traffic control by chemokines. Nat Immunol 2, 123–128 (2001). https://doi.org/10.1038/84219

[34] Seo, W., Shimizu, K., Kojo, S. et al. Runx-mediated regulation of CCL5 via antagonizing two enhancers influences immune cell function and anti-tumor immunity. Nat Commun 11, 1562 (2020). https://doi.org/10.1038/s41467-020-15375-w

[35] Perera PY, Lichy JH, Waldmann TA, Perera LP. The role of interleukin-15 in inflammation and immune responses to infection: implications for its therapeutic use. Microbes Infect. 2012 Mar;14(3):247-61. doi: 10.1016/j.micinf.2011.10.006. Epub 2011 Oct 25. PMID: 22064066; PMCID: PMC3270128.

[36] Jabri, B., Abadie, V. IL-15 functions as a danger signal to regulate tissue-resident T cells and tissue destruction. Nat Rev Immunol 15, 771–783 (2015). https://doi.org/10.1038/nri3919

[37] De La Cruz NC, Möckel M, Wirtz L, Knebel-Mörsdorf D. *Ex vivo* Human Skin Infection with Herpes Simplex Virus 1. Bio Protoc. 2022 May 5;12(9):e4411. doi: 10.21769/BioProtoc.4411. PMID: 35800458; PMCID: PMC9090582.

[38] Bhat, P., Leggatt, G., Waterhouse, N. *et al.* Interferon-*γ* derived from cytotoxic lymphocytes directly enhances their motility and cytotoxicity. *Cell Death Dis* **8**, e2836 (2017). https://doi.org/10.1038/cddis.2017.67

[40] Zhang JM, An J. Cytokines, inflammation, and pain. Int Anesthesiol Clin. 2007 Spring;45(2):27-37. doi: 10.1097/AIA.0b013e318034194e. PMID: 17426506; PMCID: PMC2785020.

[41] Melchjorsen J. Learning from the messengers: innate sensing of viruses and cytokine regulation of immunity - clues for treatments and vaccines. Viruses. 2013 Jan 31;5(2):470-527. doi: 10.3390/v5020470. PMID: 23435233; PMCID: PMC3640511.

[42] Yan Y, Hu K, Fu M,Deng X, Luo S, Tong L, Guan X, He S, Li C, Jin W, Du T, Zheng Z,Zhang M,Liu Y, Hu Q,2021. CCL19 and CCL28 Assist Herpes Simplex Virus 2 Glycoprotein D To Induce Protective Systemic Immunity against Genital Viral Challenge. mSphere6:10.1128/msphere.00058-21.https://doi.org/10.1128/msphere.00058-21

[43] Kim HC, Oh DS, Park JH, Kim H-J, Seo YB, Yoo HJ, et al. Multivalent DNA vaccine protects against genital herpes by T-cell immune induction in vaginal mucosa. *Antiviral Res* (2020) 177:104755. doi: 10.1016/j.antiviral.2020.104755

[44] Zhou Y, Wang Z, Xu Y, Zhang Z, Hua R, Liu W, et al. Optimized DNA vaccine enhanced by adjuvant IL28B induces protective immune responses against herpes simplex virus type 2 in mice. *Viral Immunol* (2017) 30(8):601–14. doi: 10.1089/vim.2017.0033

[45] Shin H, Iwasaki A. A vaccine strategy that protects against genital herpes by establishing local memory T cells. Nature. 2012 Nov 15;491(7424):463-7. doi: 10.1038/nature11522. Epub 2012 Oct 17. PMID: 23075848; PMCID: PMC3499630.

[46] Alspach E, Lussier DM, Schreiber RD. Interferon γ and Its Important Roles in Promoting and Inhibiting Spontaneous and Therapeutic Cancer Immunity. Cold Spring Harb Perspect Biol. 2019 Mar 1;11(3):a028480. doi: 10.1101/cshperspect.a028480. PMID: 29661791; PMCID: PMC6396335.

[47] Tau G, Rothman P. Biologic functions of the IFN-gamma receptors. Allergy. 1999 Dec;54(12):1233-51. doi: 10.1034/j.1398-9995.1999.00099.x. PMID: 10688427; PMCID: PMC4154595.

[48] Kotenko SV, Rivera A, Parker D, Durbin JE. Type III IFNs: Beyond antiviral protection. Semin Immunol. 2019 Jun;43:101303. doi: 10.1016/j.smim.2019.101303. PMID: 31771761; PMCID: PMC7141597.

[49] Feng E, Balint E, Vahedi F, Ashkar AA. Immunoregulatory Functions of Interferons During Genital HSV-2 Infection. Front Immunol. 2021 Aug 18;12:724618. doi: 10.3389/fimmu.2021.724618. PMID: 34484233; PMCID: PMC8416247.

[50] McNab, F., Mayer-Barber, K., Sher, A. *et al.* Type I interferons in infectious disease. *Nat Rev Immunol* **15**, 87–103 (2015). https://doi.org/10.1038/nri3787

[51] Ivashkiv, L.B. IFNγ: signalling, epigenetics and roles in immunity, metabolism, disease and cancer immunotherapy. *Nat Rev Immunol* **18**, 545–558 (2018). https://doi.org/10.1038/s41577-018-0029-z

[52] Weinberg A, Kroehl ME, Johnson MJ et al (2018) Comparative immune responses to licensed herpes zoster vaccines. J Infect Dis 218(Suppl_2):S81–S87. https://doi.org/10.1093/infdis/jiy383

[53] Sandgren, K.J., Truong, N.R., Smith, J.B., Bertram, K., Cunningham, A.L. (2020). Vaccines for Herpes Simplex: Recent Progress Driven by Viral and Adjuvant Immunology. In: Diefenbach, R., Fraefel, C. (eds) Herpes Simplex Virus . Methods in Molecular Biology, vol 2060. Humana, New York, NY. https://doi.org/10.1007/978-1-4939-9814-2_2

[54] Kak, Gunjan, Raza, Mohsin and Tiwari, Brijendra K. "Interferon-gamma (IFN-γ): Exploring its implications in infectious diseases" *Biomolecular Concepts*, vol. 9, no. 1, 2018, pp. 64-79. https://doi.org/10.1515/bmc-2018-0007

[55] Han J, Wu M, Liu Z. Dysregulation in IFN-γ signaling and response: the barricade to tumor immunotherapy. Front Immunol. 2023 May 18;14:1190333. doi: 10.3389/fimmu.2023.1190333. PMID: 37275859; PMCID: PMC10233742.

[56] Pollard KM, Cauvi DM, Toomey CB, Morris KV, Kono DH. Interferon-γ and systemic autoimmunity. Discov Med. 2013 Sep;16(87):123-31. PMID: 23998448; PMCID: PMC3934799.

[57] Boehm U, Klamp T, Groot M, Howard JC. Cellular responses to interferon-gamma. Annu Rev Immunol. 1997;15:749-95. doi: 10.1146/annurev.immunol.15.1.749. PMID: 9143706.

[58] Schoggins JW, Rice CM. Interferon-stimulated genes and their antiviral effector functions. Curr Opin Virol. 2011 Dec;1(6):519-25. doi: 10.1016/j.coviro.2011.10.008. PMID: 22328912; PMCID: PMC3274382.

[59] Gopinath, D.; Koe, K.H.; Maharajan, M.K.; Panda, S. A Comprehensive Overview of Epidemiology, Pathogenesis and the Management of Herpes Labialis. *Viruses* **2023**, *15*, 225. https://doi.org/10.3390/v15010225

[60] Loonen AJM. Putative role of immune reactions in the mechanism of tardive dyskinesia. Brain Behav Immun Health. 2023 Sep 23;33:100687. doi: 10.1016/j.bbih.2023.100687. PMID: 37810262; PMCID: PMC10550815.

[61] York, A.G., Skadow, M.H., Oh, J. *et al.* IL-10 constrains sphingolipid metabolism to limit inflammation. *Nature* 627, 628–635 (2024). https://doi.org/10.1038/s41586-024-07098-5

[62] Saraiva, M., O'Garra, A. The regulation of IL-10 production by immune cells. *Nat Rev Immunol* 10, 170–181 (2010). https://doi.org/10.1038/nri2711

[63] Trinchieri G. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. Annu Rev Immunol. 1995;13:251-76. doi: 10.1146/annurev.iy.13.040195.001343. PMID: 7612223.

[64] Ghouse, S. M., Nguyen, H., Bommareddy, P. K., & Saha, D. (2020). Oncolytic Herpes Simplex Virus Encoding IL12 Controls Triple-Negative Breast Cancer Growth and Metastasis. *Frontiers in Oncology*, *10*, 522681. https://doi.org/10.3389/fonc.2020.00384

[65] Benjamin A. Diner, Krystal K. Lum, Aaron Javitt, Ileana M. Cristea, Interactions of the Antiviral Factor Interferon Gamma-Inducible Protein 16 (IFI16) Mediate Immune Signaling and Herpes Simplex Virus-1 Immunosuppression*, Molecular & Cellular Proteomics, Volume 14, Issue 9, 2015, Pages 2341-2356, ISSN 1535-9476, https://doi.org/10.1074/mcp.M114.047068.

[66] Thompson MR, Sharma S, Atianand M, Jensen SB, Carpenter S, Knipe DM, Fitzgerald KA, Kurt-Jones EA. Interferon γ-inducible protein (IFI) 16 transcriptionally regulates type i interferons and other interferon-stimulated genes and controls the interferon response to both DNA and RNA viruses. J Biol Chem. 2014 Aug 22;289(34):23568-81. doi: 10.1074/jbc.M114.554147. Epub 2014 Jul 7. PMID: 25002588; PMCID: PMC4156042.

[67] Andrew Williams, Chemokines: CC Family,Editor(s): Sam M Janes, Encyclopedia of Respiratory Medicine (Second Edition), Academic Press, 2022, Pages 332-356, ISBN 9780081027240, https://doi.org/10.1016/B978-0-08-102723-3.00240-7.

[68] Melchjorsen J, Pedersen FS, Mogensen SC, Paludan SR. Herpes simplex virus selectively induces expression of the CC chemokine RANTES/CCL5 in macrophages through a mechanism dependent on PKR and ICP0. J Virol. 2002 Mar;76(6):2780-8. doi: 10.1128/jvi.76.6.2780-2788.2002. PMID: 11861845; PMCID: PMC135968.

[69] Manoj Thapa, Robert S. Welner, Rosana Pelayo, Daniel J. J. Carr; CXCL9 and CXCL10 Expression Are Critical for Control of Genital Herpes Simplex Virus Type 2 Infection through Mobilization of HSV-Specific CTL and NK Cells to the Nervous System[1]. *J Immunol* 15 January 2008; 180 (2): 1098–1106. https://doi.org/10.4049/jimmunol.180.2.1098

[70] Mertowska P, Smolak K, Mertowski S, Grywalska E. Immunomodulatory Role of Interferons in Viral and Bacterial Infections. Int J Mol Sci. 2023 Jun 14;24(12):10115. doi: 10.3390/ijms241210115. PMID: 37373262; PMCID: PMC10298684.

[71] Castro F, Cardoso AP, Gonçalves RM, Serre K, Oliveira MJ. Interferon-Gamma at the Crossroads of Tumor Immune Surveillance or Evasion. Front Immunol. 2018 May 4;9:847. doi: 10.3389/fimmu.2018.00847. PMID: 29780381; PMCID: PMC5945880.

[72] Willenborg DO, Fordham S, Bernard CC, Cowden WB, Ramshaw IA. IFN-gamma plays a critical down-regulatory role in the induction and effector phase of myelin oligodendrocyte glycoprotein-induced autoimmune encephalomyelitis. *J Immunol* (1996) 157:3223–7.

[73] Singh R, Kumar A, Creery WD, Ruben M, Giulivi A, Diaz-Mitoma F. Dysregulated Expression of IFN-γ and IL-10 and Impaired IFN-γ-Mediated Responses at Different Disease Stages in Patients With Genital Herpes Simplex Virus-2 Infection. *Clin Exp Immunol* (2003) 133:97–107. doi: 10.1046/j.1365-2249.2003.02183.x

[74] Danastas K, Guo G, Merjane J, Hong N, Larsen A, Miranda-Saksena M, Cunningham AL.2023.Interferon inhibits the release of herpes simplex virus-1 from the axons of sensory neurons. mBio14:e01818-23.https://doi.org/10.1128/mbio.01818-23

[75] Li Y, Song Y, Zhu L, Wang X, Richers B, Leung DYM, Bin L. Interferon Kappa Is Important for Keratinocyte Host Defense against Herpes Simplex Virus-1. J Immunol Res. 2020 Jan 3;2020:5084682. doi: 10.1155/2020/5084682. PMID: 32352019; PMCID: PMC7178474.

[76] Chew T, Taylor KE, Mossman KL. Innate and adaptive immune responses to herpes simplex virus. Viruses. 2009 Dec;1(3):979-1002. doi: 10.3390/v1030979. Epub 2009 Nov 18. PMID: 21994578; PMCID: PMC3185534.

[77] Kim HC, Oh DS, Park JH, Kim HJ, Seo YB, Yoo HJ, Jang HS, Shin J, Kim CW, Kwon MS, Jin HT, Lee SK, Oh JE, Lee HK. Multivalent DNA vaccine protects against genital herpes by T-cell immune induction in vaginal mucosa. Antiviral Res. 2020 May;177:104755. doi: 10.1016/j.antiviral.2020.104755. Epub 2020 Feb 26. PMID: 32112797.

[78] Yan Y, Hu K, Fu M, Deng X, Luo S, Tong L, Guan X, He S, Li C, Jin W, Du T, Zheng Z, Zhang M, Liu Y, Hu Q,2021.CCL19 and CCL28 Assist Herpes Simplex Virus 2 Glycoprotein D To Induce Protective Systemic Immunity against Genital Viral Challenge. mSphere6:10.1128/msphere.00058-21.https://doi.org/10.1128/msphere.00058-21

[79] Thapa M, Carr DJ. Chemokines and Chemokine Receptors Critical to Host Resistance following Genital Herpes Simplex Virus Type 2 (HSV-2) Infection. Open Immunol J. 2008;1:33-41. doi: 10.2174/1874226200801010033. PMID: 19043604; PMCID: PMC2586324.

[80] Shin, H., Iwasaki, A. A vaccine strategy that protects against genital herpes by establishing local memory T cells. *Nature* 491, 463–467 (2012). https://doi.org/10.1038/nature11522

[81] Milligan GN, Bernstein DI. Interferon-gamma enhances resolution of herpes simplex virus type 2 infection of the murine genital tract. Virology. 1997;229:259–268. doi: 10.1006/viro.1997.8441

[82] Dobbs ME, Strasser JE, Chu C-F, Chalk C, Milligan GN. Clearance of herpes simplex virus type 2 by CD8+ T cells requires gamma interferon and either perforin- or Fas-mediated cytolytic mechanisms. J Virol. 2005;79:14546–14554. doi: 10.1128/JVI.79.23.14546-14554.2005.

[83] Sherwood JK, Zeitlin L, Whaley KJ, Cone RA, Saltzman M. Controlled release of antibodies for long-term topical passive immunoprotection of female mice against genital herpes. Nat Biotechnol.

[84] Deshpande SP, Zheng M, Daheshia M, Rouse BT. Pathogenesis of herpes simplex virus-induced ocular immunoinflammatory lesions in B-cell-deficient mice. J Virol. 2000;74:3517–3524. doi:

[85] Smith TJ, Morrison LA, Leib DA2002.Pathogenesis of Herpes Simplex Virus Type 2 Virion Host Shutoff (vhs) Mutants. J Virol76:.https://doi.org/10.1128/jvi.76.5.2054-2061.2002[86] Elgadi MM, Hayes CE, Smiley JR. The herpes simplex virus vhs protein induces endoribonucleolytic cleavage of target RNAs in cell extracts. J Virol. 1999 Sep;73(9):7153-64. doi: 10.1128/JVI.73.9.7153-7164.1999. PMID: 10438802; PMCID: PMC104239

[87] Lane TE, Liu MT, Chen BP, et al. A central role for CD4+ T cells and RANTES in virus-induced central nervous system inflammation and demyelination. J Virol. 2000;74(3):1415–1424. doi: 10.1128/jvi.74.3.1415-1424.2000.

[88] Chiliveru, S., S. H. Rahbek, S. K. Jensen, S. E. Jørgensen, S. K. Nissen, S. H. Christiansen, T. H. Mogensen, M. R. Jakobsen, L. Iversen, C. Johansen, S. R. Paludan. 2014. Inflammatory cytokines break down intrinsic immunological tolerance of human primary keratinocytes to cytosolic DNA. J. Immunol. 192: 2395–2404.

[89] C.N. Sodroski,& D.M. Knipe, Nuclear interferon-stimulated gene product maintains heterochromatin on the herpes simplex viral genome to limit lytic infection, Proc. Natl. Acad. Sci. U.S.A. 120 (45) e2310996120, https://doi.org/10.1073/pnas.2310996120 (2023).

[90] Zhang M, Fu M, Li M, Hu H, Gong S, Hu Q. Herpes Simplex Virus Type 2 Inhibits Type I IFN Signaling Mediated by the Novel E3 Ubiquitin Protein Ligase Activity of Viral Protein ICP22. J Immunol. 2020 Sep 1;205(5):1281-1292. doi: 10.4049/jimmunol.2000418. Epub 2020 Jul 22. PMID: 32699158.

[91] Kak, Gunjan & Raza, Mohsin & Tiwari, Brijendra. (2018). Interferon-gamma (IFN-γ): Exploring its implications in infectious diseases. Biomolecular Concepts. 9. 64-79. 10.1515/bmc-2018-0007.

[92] Han J, Wu M, Liu Z. Dysregulation in IFN-γ signaling and response: the barricade to tumor immunotherapy. Front Immunol. 2023 May 18;14:1190333. doi: 10.3389/fimmu.2023.1190333. PMID: 37275859; PMCID: PMC10233742.

[93] Pollard KM, Cauvi DM, Toomey CB, Morris KV, Kono DH. Interferon-γ and systemic autoimmunity. Discov Med. 2013 Sep;16(87):123-31. PMID: 23998448; PMCID: PMC3934799.

[94] Miller CH, Maher SG, Young HA. Clinical Use of Interferon-gamma. Ann N Y Acad Sci. 2009 Dec;1182:69-79. doi: 10.1111/j.1749-6632.2009.05069.x. PMID: 20074276; PMCID: PMC6574079.

[95] Melchjorsen J, Matikainen S, Paludan SR. Activation and evasion of innate antiviral immunity by herpes simplex virus. Viruses. 2009 Dec;1(3):737-59. doi: 10.3390/v1030737. Epub 2009 Nov 5. PMID: 21994567; PMCID: PMC3185509.

[96] Walker FC, Sridhar PR, Baldridge MT. Differential roles of interferons in innate responses to mucosal viral infections. Trends Immunol. 2021 Nov;42(11):1009-1023. doi: 10.1016/j.it.2021.09.003. Epub 2021 Oct 7. PMID: 34629295; PMCID: PMC8496891.

[97] Vilgelm AE, Richmond A. Chemokines Modulate Immune Surveillance in Tumorigenesis, Metastasis, and Response to Immunotherapy. Front Immunol. 2019 Feb 27;10:333. doi: 10.3389/fimmu.2019.00333. PMID: 30873179; PMCID: PMC6400988.

[98] Lazzaro BP, Sceurman BK, Clark AG. Genetic basis of natural variation in D. melanogaster antibacterial immunity. Science. 2004 Mar 19;303(5665):1873-6. doi: 10.1126/science.1092447. PMID: 15031506.

[99] Kwiatkowski DP. The complexity of genetic variation in a simple immune system. Trends Genet. 2005 Apr;21(4):197-9. doi: 10.1016/j.tig.2005.02.002. PMID: 15797611.

[100] Harandi AM, Svennerholm B, Holmgren J, Eriksson K. Interleukin-12 (IL-12) and IL-18 are important in innate defense against genital herpes simplex virus type 2 infection in mice but are not required for the development of acquired gamma interferon-mediated protective immunity. J Virol. 2001 Jul;75(14):6705-9. doi: 10.1128/JVI.75.14.6705-6709.2001. PMID: 11413339; PMCID: PMC114395.

[101] Malmgaard L, Paludan SR, Mogensen SC, Ellermann-Eriksen S. Herpes simplex virus type 2 induces secretion of IL-12 by macrophages through a mechanism involving NF-kappaB. J Gen Virol. 2000 Dec;81(Pt 12):3011-3020. doi: 10.1099/0022-1317-81-12-3011. PMID: 11086132.

[102] Sin JI, Kim JJ, Arnold RL, Shroff KE, McCallus D, Pachuk C, McElhiney SP, Wolf MW, Pompa-de Bruin SJ, Higgins TJ, Ciccarelli RB, Weiner DB. IL-12 gene as a DNA vaccine adjuvant in a herpes mouse model: IL-12 enhances Th1-type CD4+ T cell-mediated protective immunity against herpes simplex virus-2 challenge. J Immunol. 1999 Mar 1;162(5):2912-21. PMID: 10072541.

[103] Ashkar AA, Rosenthal KL.2003.Interleukin-15 and Natural Killer and NKT Cells Play a Critical Role in Innate Protection against Genital Herpes Simplex Virus Type 2 Infection. J Virol77:.https://doi.org/10.1128/jvi.77.18.10168-10171.2003

[104] Gill N, Ashkar AA. Adaptive immune responses fail to provide protection against genital HSV-2 infection in the absence of IL-15. Eur J Immunol. 2007 Sep;37(9):2529-38. doi: 10.1002/eji.200636997. PMID: 17668897.

[105] Perera PY, Lichy JH, Waldmann TA, Perera LP. The role of interleukin-15 in inflammation and immune responses to infection: implications for its therapeutic use. Microbes Infect. 2012 Mar;14(3):247-61. doi: 10.1016/j.micinf.2011.10.006. Epub 2011 Oct 25. PMID: 22064066; PMCID: PMC3270128.

[106] Hu XD, Chen ST, Li JY, Yu DH, Yi Z, Cai H. An IL-15 adjuvant enhances the efficacy of a combined DNA vaccine against Brucella by increasing the CD8+ cytotoxic T cell response. Vaccine. 2010;28:2408–2415. doi: 10.1016/j.vaccine.2009.12.076.

[107] Faivre, N., Verollet, C. & Dumas, F. The chemokine receptor CCR5: multi-faceted hook for HIV-1. *Retrovirology* **21**, 2 (2024). https://doi.org/10.1186/s12977-024-00634-1

[108] Aldinucci D, Colombatti A. The inflammatory chemokine CCL5 and cancer progression. Mediators Inflamm. 2014;2014:292376. doi: 10.1155/2014/292376. Epub 2014 Jan 2. PMID: 24523569; PMCID: PMC3910068.

[109] Unterholzner L, Keating SE, Baran M, Horan KA, Jensen SB, Sharma S, Sirois CM, Jin T, Latz E, Xiao TS, Fitzgerald KA, Paludan SR, Bowie AG. IFI16 is an innate immune sensor for intracellular DNA. Nat Immunol. 2010 Nov;11(11):997-1004. doi: 10.1038/ni.1932. Epub 2010 Oct 3. PMID: 20890285; PMCID: PMC3142795.

[110] A.P. Costa-Pereira, S. Tininini, B. Strobl, T. Alonzi, J.F. Schlaak, H. Is'harc, I. Gesualdo, S.J. Newman, I.M. Kerr, & V. Poli, Mutational switch of an IL-6 response to an interferon-γ-like response, Proc. Natl. Acad. Sci. U.S.A. 99 (12) 8043-8047, https://doi.org/10.1073/pnas.122236099 (2002).