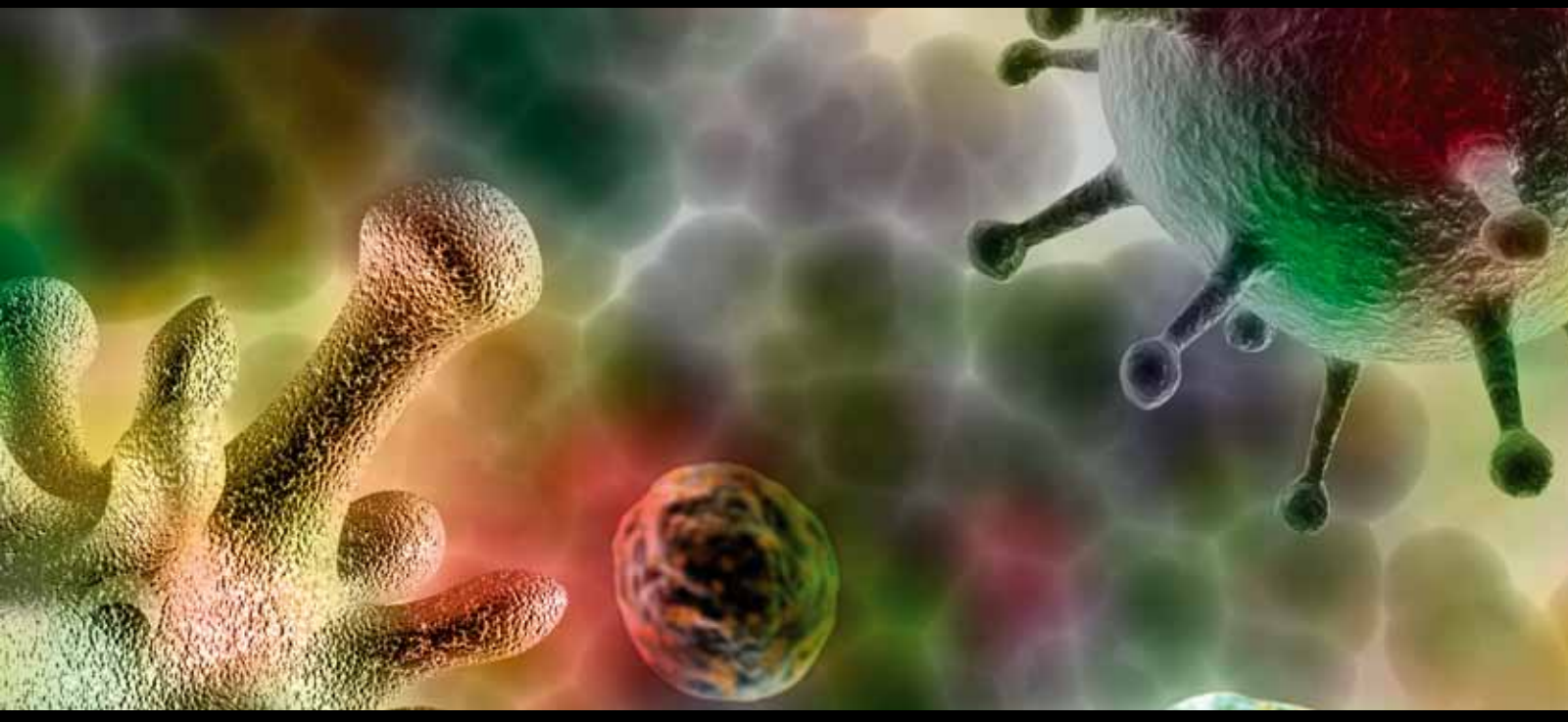
Advances in Virology

Virus Immune Evasion: New Mechanism and Implications in Disease Outcome

Guest Editors: Rika Draenert, John Frater, and Julia G. Prado



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Editorial Board

Contents

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Editorial

Virus Immune Evasion: New Mechanism and Implications in Disease Outcome

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The artists of immune evasion in nature are viruses, as ancient as their organic counterparts, these infectious pseudoorganisms have coevolved coupling human and animal evolution during millions of years. Thus, virus replication is based on an efficient recruitment of cell functions through interactions between cellular and viral proteins. As consequence, during their long evolutionary race the immune system has specialized to control pathogens, and virus has specialized to counteract immune responses favour in the battle by their rapid adaptation against environmental changes.

In this special issue, we summarize some of the current knowledge in immune evasion strategies developped by both DNA and RNA viruses. Virus explored and exploits multiple components of the cellular biogenesis and immune signalling. Thus, virus release and infection are enhanced through the convergence of viral and cellular pathways as summarized by T. Wurdinger et al. Alternative mechanisms of chemokine receptors capture (A. G. Jensen et al.), interference of immune signalling cascades and natural killer responses (G. J. Kotwal et al. and C. I. Odom et al.) are some of the strategies acquire for herpesvirus, poxvirus or retrovirus among others (S. N. Schelkunov et al.), to counteract both innate and adaptative host immunity.

Additionally, we cannot forget that globally virus are the causative agent of multiple diseases leading to worldwide pandemics and associated with multiple human malignancies (S. Chapenko et al.). The aim of this special issue is to put into perspective the need to understand virus immune escape based on a deep knowledge of viral strategies in order to develop new vaccines and therapeutic approaches (B. Reinhart et al.). In this context, the design of new interventions should be truly innovative and effective in order to reduce mortality and mobility rates associated with viral infection in humans.

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Review Article

Viral Infection: An Evolving Insight into the Signal Transduction Pathways Responsible for the Innate Immune Response

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The innate immune response is initiated by the interaction of stereotypical pathogen components with genetically conserved receptors for extracytosolic pathogen-associated molecular patterns (PAMPs) or intracytosolic nucleic acids. In multicellular organisms, this interaction typically clusters signal transduction molecules and leads to their activations, thereby initiating signals that activate innate immune effector mechanisms to protect the host. In some cases programmed cell death—a fundamental form of innate immunity—is initiated in response to genotoxic or biochemical stress that is associated with viral infection. In this paper we will summarize innate immune mechanisms that are relevant to viral pathogenesis and outline the continuing evolution of viral mechanisms that suppress the innate immunity in mammalian hosts. These mechanisms of viral innate immune evasion provide significant insight into the pathways of the antiviral innate immune response of many organisms. Examples of relevant mammalian innate immune defenses host defenses include signaling to interferon and cytokine response pathways as well as signaling to the inflammasome. Understanding which viral innate immune evasion mechanisms are linked to pathogenesis may translate into therapies and vaccines that are truly effective in eliminating the morbidity and mortality associated with viral infections in individuals.

1. Introduction

The innate immune system is as ancient as the bacterial immune response to bacteriophages. As the nature and complexity of viral innate immune evasion mechanisms evolved, so has the innate—and eventually adaptive—immune response to these mechanisms. The innate immune response in mammals is initiated by the interaction of stereotypical pathogen components with germ-line encoded receptors. In some cases, signal transduction pathways are stimulated in sentinel cells, such as macrophages and dendritic cells. Stimulation of these signaling pathways promptly activates innate effector mechanisms to protect the host; these innate immune signals also activate antigen-presenting cells that are critical to the eventual adaptive immune response of the host [1]. In this paper we will summarize findings in the innate immune system that are relevant to viral pathogenesis and outline the evolution of viral mechanisms that suppress innate immunity in mammalian hosts.

2. The Innate Immune System

The receptors of the innate immune system are germline encoded and include the nucleotide-binding domain leucine-rich repeat containing receptors, the Toll-like receptors (TLRs), and the RIG-I-like receptors (RLRs). The RLRs are cytosolic sensors of pathogen RNA and include proteins encoded by the retinoic acid-inducible gene-I (RIG-I) [2], the melanoma differentiation-associated gene 5 (MDA5) [3, 4], and the laboratory of genetics protein 2 (LGP2) [4] and DDX3, which is thought to associate with RIG-I [5]. The helicase domains of RLRs detect the cytosolic RNA of microbial pathogens, generating signals that drive

production of cytokines and interferons. Helicases are ATP-dependent enzymes that unidirectionally translocate along a transcript thereby dissociating nucleic acid duplexes [6]. The RIG-I and MDA5 RLRs play critical roles in the recognition of foreign RNA and in the response to many viral pathogens. MDA5 and RIG-I contain a DExD/H-box RNA helicase domain and caspase activation and recruitment domains (CARDs). RIG-I recognizes 5′-triphosphate RNA, and MDA5 can recognize complex webs of pathogen RNA, comprised of both viral single-stranded and double-stranded RNA [2]. The LGP2 RLR protein was found to lack a CARD domain and was originally identified as a dominant negative inhibitor of RIG-I signaling [7]. Under some circumstances, though, it appears LGP2 can stimulate RLRs such as MDA5 and RIG-I [8]. CARD engagement leads to interaction with a protein known as mitochondrial antiviral signaling protein (MAVS) that is alternatively designated CARDIF, HELICARD, or IPS-1 (referred to here as MAVS) [9, 10]. Subsequently, upon oligomerization, MAVS signals to members of the IKK family of kinases that are critical for the innate immune response [10]. Thus MAVS induces IKKα and IKKβ stimulation that leads to translocation of NF-κB, as well as IKKε/TBK1 stimulation that leads to translocation of IRF-3. These transcription factors stimulate production of cytokines, other innate immune response proteins, and type I interferons [4].

Extracytosolic innate immune sensing of pathogens is mediated via the TLRs. Humans are known to encode ten TLRs which are each involved in the recognition of different pathogen-associated molecular constituents [11]. The TLRs are transmembrane receptors found on the cell surface and/or associated with endocytic vesicles [11]. Thus, they are ideally situated to detect extracytosolic pathogens. For example, TLR4 is required for the recognition of Gram-negative bacterial lipopolysaccharide (LPS, or endotoxin) while TLR3 is able to recognize dsRNA, a signature compound common in the lifecycle of many viruses, while TLR7 and 8 recognize ssRNA [12]. Toll/IL-1 interacting receptor (TIR) adapters ultimately stimulate IkB family kinases (IKKs) often via transducing proteins such as IRAKs and TRAFs, thereby mediating signaling originally induced by engagement of TLRs that ultimately activates NF-κB and IRF3 [13].

Two classical IKKs, IKKα and IKKβ, are critical for NF-κB activation. They function, in large part, by phosphorylating the inhibitors of NF-κB, known as IkBs. Once phosphorylated, IkBs are ubiquitinated and degraded. This allows NF-κB subunits to translocate to the nucleus and activate target gene expression. NF-κB is critical for driving the expression of numerous cytokines, chemokines, and costimulatory molecules, creating an inflammatory response [14]. On the other hand, the two nonclassical IKK family members, IKKε and TBK1, are implicated in IRF3 activation. In particular, they are believed to directly phosphorylate several serine residues within the C-terminal activation domain of IRF3. Once phosphorylated, IRF3 dimerizes and translocates to the nucleus where it activates target gene expression [15]. IRF3 activation is critically important for the activation of type I interferons, either directly [16] or via an autocrine/paracrine loop [17, 18]. Type I interferons, in turn, are capable of inducing a significant antiviral response in the host [2, 13, 19].

Cells also encode cytosolic DNA sensors which detect DNA, which is not typically present in the cytosol and thus a pattern whose recognition signals the presence of viral nucleic acids. Nucleotide oligomerization domain-like receptor proteins (NLRs) are implicated in the intracytosolic recognition of sterile inflammatory instigators, such as urate crystals, intracytosolic DNA, or viral RNA. One such NLR, nucleotide oligomerization domain-like receptor protein 3 (NLRP3) is an inflammasome component that signals to the apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) to induce the clustering-induced self-processing of procaspase 1 into caspase 1 which then digests the precursor form of pro-IL-1β and pro-IL-18 to permit release of the inflammatory cytokines IL-1β and IL-18 from the cell [20]. Similar inflammatory pathways are triggered by engagement of other cytosolic DNA sensors such as AIM-2, another NLR that ultimately induces cleavage of procaspase 1 into caspase 1. Aim-2 can detect the molecular patterns of intracellular hazards such as pathogen DNA, particularly that of poxviruses [21]. Other NOD proteins which are alternatively designated NACHT, LRR, and PYD function as sensors of toxic intracellular molecules including cytosolic DNA [22]. Thus the NLRs represent examples of cytosolic DNA sensors capable of inducing an inflammatory antiviral response.

Another antiviral cytosolic sensor is the DNA-dependent activator of interferon (DAI), which binds B- and Z-form DNA, thereby recognizing intracytosolic viral DNA. Signals from such sensors are transduced by known innate immune kinases such as TBK1, which interacts with a protein known as stimulator of interferon genes (STINGs) to activate NF-κB and IRF3 signaling [10, 23, 24]. Finally, it is assumed that there is at least one other pathway for the detection of the dsDNA of microbes, based in part on DNA sensing in cells despite absence of the DAI pathway [25]. The known receptors for viral DNA ultimately induce interferons, cytokines, and programmed cell death pathways.

Apoptosis is the programmed death of dangerous or unnecessary cells, for example, virally infected, aging, or malignant cells. It is thus one of the most ancient forms of innate immunity. Certain cellular bcl-2 proteins mediate resistance to programmed cell death (apoptosis) [50–52], typically via interaction with proapoptotic bcl-2-related proteins [52]. Human bcl-2 also leads to increased nuclear translocation of the transcription factor, NF-κB [53–55], which typically promotes cell survival [14, 56, 57]. Other cellular bcl-2 proteins promote cell death in response to harmful stimuli such as viral infection. Other effectors of programmed cell death are caspases. Cleavage of cellular caspases and/or loss of mitochondrial integrity promote cell death in the face of many stimuli including viral infection. Still other death programs include pyroptosis—the death of cells following activation of the PYRIN domains and IL-1 release. Thus, in the absence of viral innate immune evasion, apoptosis provides an antiviral mechanism for the elimination of virally infected cells.

Table 1: Examples of parallel evolution of viral innate immune evasion mechanisms.

(a) Bacteria

Mechanism	Viral evasion strategy	Virus protein
CRISPER	Genetic variation of DNA	DNA polymerase [26]
Psp-induced signaling	Unknown (Psp genetic variation)	Unknown
Restriction/methylation	Methylation of viral target DNA	Acquired bacterial methylase [26]
Apoptosis	Lysogeny/tolerance	Phage lysis gene regulation [27]

(b) *Drosophila*

Mechanism	Evasion strategy	Viral protein
DICER1	Genetic variation of DNA	Virus encodes miRNAs [28]
Vago	IkB, Jak/Stat inhibitors	Viral IkB, unknown inhibitor of Jak/STAT [29]
DICER2	Binding of ds RNA	FHV-B2 protein [30]
Apoptosis	Inhibitor of apoptosis (IAP)	Baculovirus p35 [31, 32]

(c) Mammalian systems

Mechanism	Evasion strategy	Viral protein
Interferon signaling	dsRNA binding	Influenza virus NS1 [33], VACV E3 (also inhibits DNApol III DNA sensing) [34]
	Inhibition of signaling	VACV N1 family [5, 35–37], HCV NS3A/4 [38], influenza virus NS1 [33], HCV core proteins inhibit Jak/Stat signaling [39], HCV NS5A inhibits MyD88 [40], HIV Vif, and Vpr degrade IRF3 [37]
	Inhibition of IFN binding	VACV soluble IFN alpha/beta receptors [41]
Viral RNA/DNA sensing	Inhibitors of RIG-I	HCV NS3A/4 proteolytic cleavage of MAVS [38, 42]
	Inhibitors of MDA5, LGP2	Paramyxovirus V proteins [43]
	Inhibitors of DDX3	VACV K7 [5]
	Inhibitors of the AIM2/NLRP3 Inflammasome	KSHV vNLR [44], myxoma virus M013 [45]
	Inhibitors of proteins activated downstream of the AIM2/NLRP3 inflammasome	Cowpox virus crmA VACV SPI-2, sIL-1βR, and sIL-18R [41]
	DAI	EBV EBERmiRNAs [25]
Programmed cell death	Viral inhibitor of apoptosis (IAP)	KSHV vFLIP [46]
	Viral bcl-2s	EBV bhrf1 and balf1 [47, 48], KSHV orf16 [49]
	Blockade of IL-1-mediated pyroptosis	Poxvirus crmA, sIL-1β, sIL18 [41]

3. Evolution of the Antiviral Innate Immune Response: Different Genes, Recurring Themes

Prokaryotic organisms encode primordial proteins that recognize the molecular patterns (e.g., specific sequences of DNA of bacteriophages) from pathogens (i.e., bacteriophages) and thus can be considered to possess a primitive innate immune system. Although the mechanisms of innate immunity in bacteria differ radically from those of higher organism, four principles of innate immunity are preserved in several mechanisms (Table 1). First, the clustered regularly space short palindromic repeats (CRISPERs) of bacteria and archaea encode a series of palindromic sequences that target pathogen DNA and suppress their transcription in a way similar to the antiviral action of microRNAs of *Drosophila* [58, 59]. Second, following exposure of prokaryotes to bacteriophages, the phage shock protein (Psp) signaling pathway

involves an unknown sensor and signal transduction by the leucine zipper protein PspB. PsP signaling is initiated in response to loss of cell membrane integrity induced by stresses such a bacteriophage infection [60]. This is similar in principle to the enhanced cell membrane integrity mediated by interferon in the mammalian antiviral response [2, 13, 19]. A third conserved principle is intracytosolic nucleic acid recognition (analogous to mammalian RLRs or *Drosophila* DICER) that triggers an innate immune response. In bacteria, restriction endonucleases recognize and digest bacteriophage nucleic acids, while specificity of this response is maintained by bacterial methylation of its native DNA. Fourth, programmed death of bacteriophage-infected bacteria induced by the MazF protein can pre-empt spread of viral infection, as is true of proapoptotic proteins in higher organisms (reviewed in [27]). Bacteriophage mechanisms to evade the bacterial innate immune pathways include rapid mutation to generate DNA sequence diversity that evades CRISPER, acquisition of host bacterial methylases to mask restriction sites in bacteriophage DNA [26], and programmed cell death resistance [27]. A bacteriophage mechanism to evade PsP signaling has not been reported, although it is tempting to speculate that the rapid mutation observed during bacteriophage infection might avoid detection by the Psp pathway. Although the details and evolution of innate immune mechanisms in bacterial cells are highly divergent from multicellular organisms (Table 1), the principal functional attributes of innate immune recognition and viral evasion are remarkably conserved, especially in the invertebrate innate immune responses.

Drosophila lack an adaptive immune system; thus, they are ideal model organisms to study innate immunity since they possess a complex innate immune system (Table 1). Viral mechanisms for suppression of innate immunity in *Drosophila* have been reviewed recently and will be discussed only briefly here [29]. In contrast to mammalian cells, *Drosophila* does not encode NOD proteins. It has been suggested that *Drosophila* TLRs encoded recognize PAMPs of viruses that are tropic for *Drosophila* [61]. Unlike mammals, *Drosophila* rely heavily on RNA interference as a defense against viruses. The protein DICER2 is a helicase/endonuclease that is related to the RIG-I-like helicase of mammals [62]. DICER2 has two effector functions; the first initiates a cascade of endonucleolytic cleavage of viral RNAs that mediate gene silencing, and the second is a RIG-I-like signaling activity of DICER2 whereby DICER-2 mediates induction of the antiviral genes, such as *Vago* [62]. A distinct protein, DICER1, cleaves isolated miRNAs that subsequently suppress transcription of viral RNA just as mammalian DICER2 does [58, 59]. To evade this innate immune defense, the *Flockhouse* virus encodes the dsRNA-binding protein B2 that inhibits dsRNA recognition by DICER1 and DICER2 in *Drosophila* [30]. Furthermore, viruses inhibit the function of inhibitors of kappaB (I-κB) translocation to prevent signaling initiated by *Drosophila* TLRs [61], but not the Jak/Stat antiviral defense pathway in *Drosophila*. Other viral proteins that act in signal transduction are thought to mediate the production of antiviral peptides, including the principal *Drosophila* gene induced by viral infection that is *Vago,* which encodes a 14 kilodalton cysteine-rich polypeptide [62]. *Vago* is thought to be, in principle, analogous to interferons as it is a virus-induced protein critical to control viral infection. Finally, viral innate immune evasion proteins encoded by baculovirus inhibit the function of apoptotic pathways [31, 32, 63, 64]. Although there are parallels between the principles of innate immune defense against viruses between bacteria and *Drosophila* (Table 1), the innate immune responses of *Drosophila* more closely resemble those of the mammalian antiviral innate immune response.

4. Viral Evasion of Host Defenses: Highlighting Critical Components of the Mammalian Innate Immune Response

4.1. DNA Viruses. The *Poxviridae* are large enveloped DNA viruses that replicate in the cytoplasm. Vaccinia virus (VACV) is a robust poxviral vaccine originally used to eradicate smallpox. Poxviruses encode approximately 180 genes. About 80 genes are essential for replication in tissue culture, whereas 100 encode virulence proteins, such as decoy receptors for IL-1, TNF-α, and interferons. These virulence proteins (Table 1, bold text, and Figure 1) interdict innate immune signaling by preventing receptor engagement at the cell surface [65]. Moreover, the pox virus proteins, **E3** and **K3**, bind dsRNA in the cytoplasm, reducing type I interferon production and, in the case of **E3**, preventing activation of the dsRNA-dependent protein kinase PKR [34].

Other vaccinia virus proteins have been characterized as inhibitors of innate immune intracellular signal transduction (Figure 1). For example the VACV **N1** family of ten bcl-2 like proteins inhibits NF-κB signaling [37]. Of the proteins characterized to date, N1 is the most robust VACV virulence factor, increasing replication 10,000-fold, inhibiting NF-κB, IRF3 and apoptotic signaling [35, 66, 67]. **A52** inhibits NF-κB and increases p38 kinase activity [68]. **A46** inhibits NF-κB and IRF3 signaling [36]. And **K7** inhibits IRF3 and NF-κB signaling by binding to DDX3 and preventing MAVS signaling to TBK1 [5]. It is unclear what the role of N1's anti-apoptotic function is as VACV *already* encodes a vbcl-2 (F1), that, unlike N1, is critical for viral survival *in vitro*. Thus, the antiapoptotic potential of the N1 vbcl-2 reconciles the absence of cell death despite N1 inhibition of NF-κB. While no direct inhibitor of the inflammasome has been detected in vaccinia virus, the poxvirus serpins **SPI-1** and **crmA** inhibit caspase 1 activity downstream of the inflammasome [69] and another poxvirus, myxoma virus encodes the **M013** PYRIN domain containing protein that inhibits signaling by the inflammasome by interrupting association of NLRP3 and ASC ([45], see Figure 2). Thus poxviruses inhibit many aspects of the two-signal inflammasome inflammatory pathway by inhibiting pro-IL-1β and pro-IL-18 production by the IKK pathway and cleavage by the inflammasome (Figure 2). Additionally, as outlined in Figure 1, IKK complex signaling to TNF-α, IFN, and other cytokines is impaired by viral innate immune evasion proteins.

The gammaherpesviruses encode proteins that highlight the role of antiapoptotic factors in innate immune evasion.

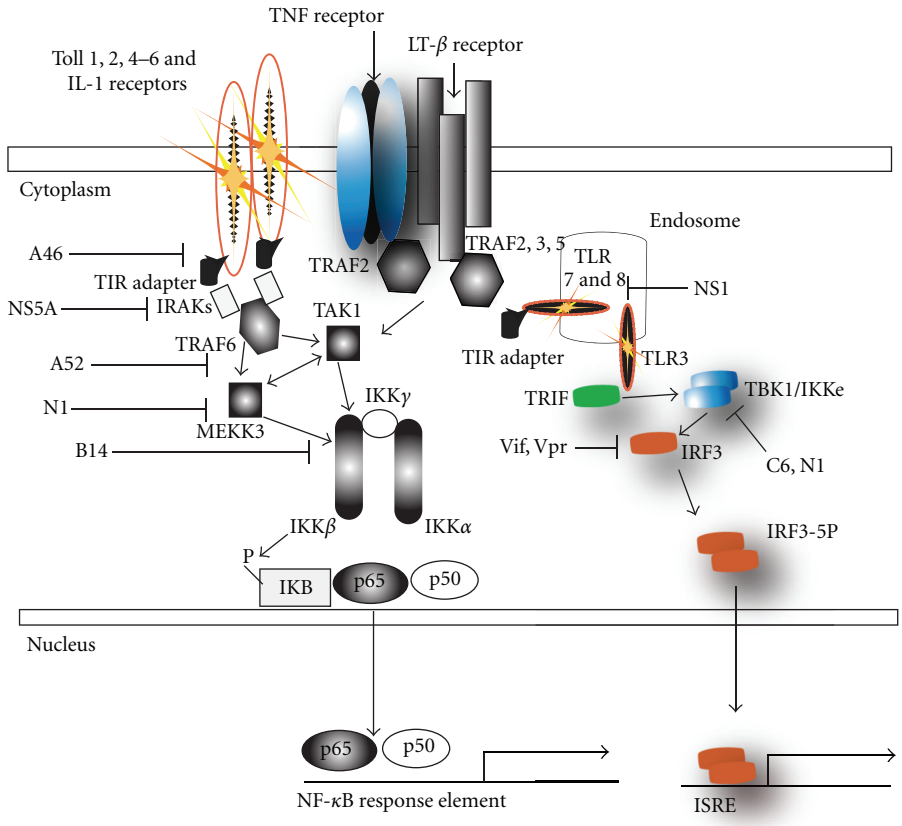

Figure 1: Signaling by the innate immune system that is inhibited by several viral proteins. This model depicts the salient features of TLR-induced NF-κB and IRF3 induction. Several poxviral proteins N1, C6, A14 A46, and A52 inhibit the activation of NF-κB and/or IRF3 signaling pathways, by interacting with and inhibiting the activity of the classical IKK complex (IKKα/β/γ) as well as the nonclassical IKKε/TBK1 complex. HCV protein NS5A inhibits TIR signaling by MyD88, its NS3/4A digests MAVS to inhibit RLR signaling, and its core protein inhibits Jak/Stat signaling. Finally, HIV Vif and Vpr degrade IRF3.

Herpesviruses cause a latent, life-long infection. During latent infection herpesvirus antigens are principally present in the nucleus thereby evading recognition by the cytosolic and extranuclear membrane-associated components of the innate immune system. The apoptotic mechanisms of the innate immune system affect the elimination of herpesvirus-infected cells. Two human gammaherpesviruses, Epstein Barr Virus (EBV) and Kaposi's Sarcoma Herpesvirus (KSHV) have evolved several mechanisms that induce latent infection and thus inhibit the apoptotic innate immune response (LMP1 and LANA1). Recent studies of recombinant EBV containing deletions of the genes encoding both the antiapoptotic vbcl-2, BHRF1 [47], and a second EBV bcl-2, BALF1 [48, 70, 71], have revealed that deletion of both EBV bcl-2 homologs dramatically increases the survival of cells undergoing EBV infection [72]. Finally, the KSHV gammaherpes orf63 has been shown to encode a viral NLR (**vNLR**, Figure 2) that inhibits NLRP1, NLRP3, and NOD2 function, permitting persistent KSHV infection [45].

Viral caspase inhibitors are believed to neutralize immune responses of the host that activate the caspase pathway of apoptotic cell death (although it is logical to hypothesize that viral caspase 1 inhibitors also inhibit the inflammasome). Three different viral proteins inhibit the caspase pathway of apoptosis: (1) the serpins of the poxviruses, exemplified by crmA, a caspase inhibitor encoded by the cowpox virus genome [69], and (2) the baculovirus p35 caspase inhibitor protein [73]. Finally, the v-FLIPs, which are expressed by the gammaherpesviruses equine herpesvirus 4 and Kaposi's Sarcoma Herpesvirus (KSHV/HHV-8) [46] inhibit apoptosis by competing with caspase 8 (FLICE) for binding to the death effector domains of adaptor proteins of death receptors (reviewed by [74]). Inhibition of the activation of the caspase cascade that would otherwise follow oligomerization of death receptors prevents apoptosis induced by the cascade of proteases that eventually induce cellular self-digestion [75]. Inhibition of caspases is thus another evolutionarily conserved mechanism for viruses to avoid the apoptotic host innate immune response. In addition, vFLIPs dysregulate the function of IKK-γ, thereby activating the IKK complex-mediated dissociation of IkB from NF-κB and subsequent NF-κB signaling [76]. Activation of the IKK cascade that would otherwise promote innate immune signaling activates KSHV replication and promotes KSHV-transformed cell survival. In the case of this KSHV mechanism, signaling by the classical innate immune

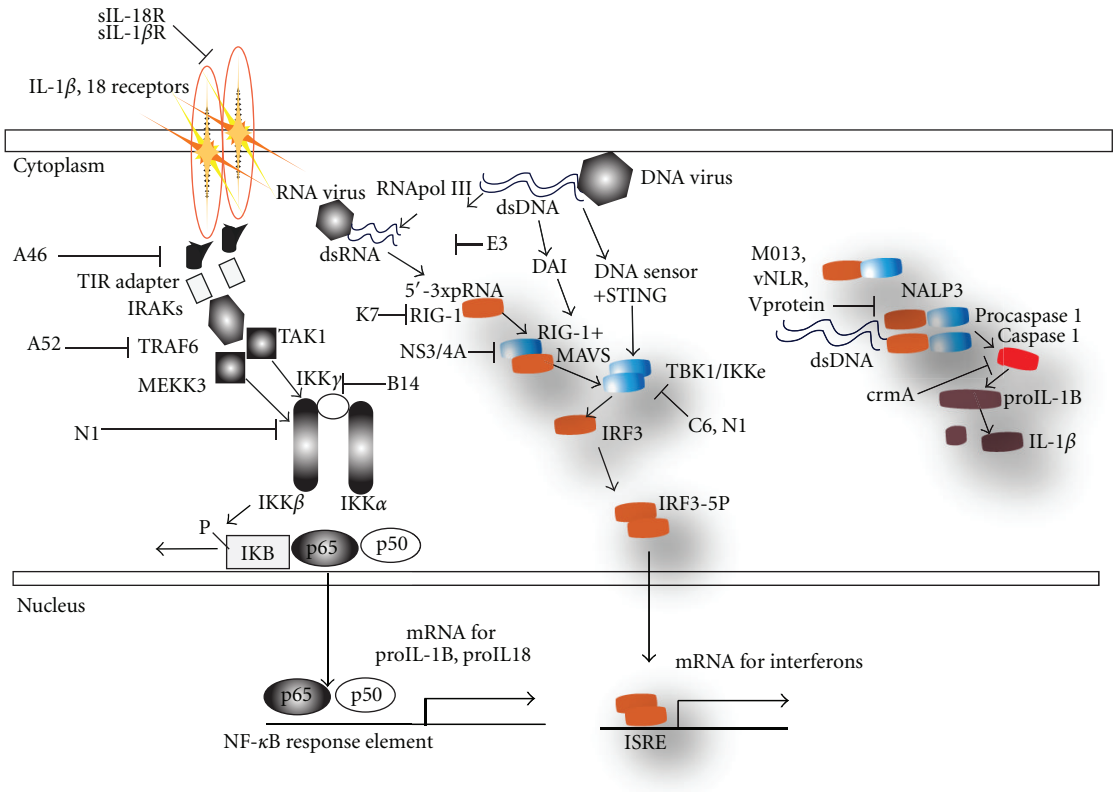

Figure 2: Viral proteins inhibit nucleic acid receptors of the intracytosolic innate immune response. Viruses inhibit each of the two signals that initiate the inflammasome activation process. The first signal—IL-1β and/or IL-18 binding and activation of the TLR/IL-1β receptor pathway—is inhibited by soluble IL-1β and IL-18 (from VACV); downstream, inhibitors of signaling to NF-κB (from VACV or HCV) repeatedly target this important antiviral pathway that optimally requires NF-κB translocation leading to the production of pro-IL-1β and pro-IL-18. Second, the inflammasome processes these pro-IL-1β and pro-IL-18 proteins via caspase-1 that is itself processed upon clustering mediated at the NLRP3 inflammasome upon detection of intracytosolic pathogens. This leads to IL-1β and IL-18 production and release that activates the IL-1β/IL-18 pathway in an autocrine manner, as well as the innate and adaptive immune response. Inflammasome activation is inhibited by myxoma virus M013, measles viruses V protein, and KSHV vNLR. Finally signaling to IRF3 by intracytosolic DNA or RNA is inhibited at the level of MAVS by HCV's NS3/4A and at the level of TBK1 by VACV C6 and N1 (Figure 1).

response pathway is perturbed by KSHV protein, allowing KSHV to escape the apoptotic host innate immune response.

4.2. RNA Viruses. Hepatitis C Virus (HCV) is a single-stranded, positive-sense RNA virus that encodes 10 proteins. HCV typically induces a life-long infection via successfully evading the adaptive and innate immune responses. The many HCV proteins that possess dual functions in both replication and innate immune evasion likely reflect the limited number of HCV. Surprisingly for the small size of its genome, HCV shares mechanisms of innate immune evasion with much larger DNA viruses such as the poxviruses. Poxvirus nucleoside triphosphate phosphohydrolase I (NPH-I) is absolutely essential for VACV mRNA transcription and VACV replication, yet simultaneously NPH-I inhibits the interferon response [77]. The HCV NS3/4A protease/helicase encodes a helicase that suppresses the IFN-β promoter independently of **NS3/4A** proteolytic destruction of innate immune signaling components such as TRIF that activate the IFN-β [42]. A recombinant RLR engineered to encode only a helicase domain is a dominant negative inhibitor of RLR-driven interferon (IFN)-β promoter activity. This dominant negative RLR lacks a signaling domain [7]. Viral helicases inherently lack signaling domains, and thus viral helicases structurally resemble a dominant negative RLR and might act as RLR antagonists. HCV innate immune evasion mechanisms also include the proteolytic destruction of MAVS by the protease component of the NS3/4A protein. The destruction of MAVS, which transduces signals from the RLRs, therefore inhibits signaling to IRF3 via TBK1, blocking the interferon response. HCV core protein expression correlates with impaired signaling of the Jak/Stat pathway to IFN-α/β, although the mechanism for this is still being defined [78]. The HCV core protein inhibits TLR signaling through its chronic stimulation of TLR2 resulting in TLR hyporesponsiveness [79], HCV core protein binds STAT1, and HCV infection leads to STAT1 degradation, which inhibits the antiviral signaling in the Jak/Stat pathway [80]. Finally HCV protein **NS5A** inhibits recruitment of IRAK to the MyD88

TIR adapter [40] and may inhibit interferon production via NS5A suppression and the phosphorylation of eIF2 by the PKR kinase [40]. Thus, HCV inhibits several types of innate immune signaling via the action of only a few proteins.

Influenza viruses are enveloped RNA viruses with a negative-sense, single-stranded segmented genome. Influenza virus is an extremely virulent respiratory pathogen, and influenza virus possesses distinct innate immune evasion mechanisms that are critical for its pathogenesis. The nonstructural protein-1 (NS1) functions to inhibit the host interferon response [81], thus inhibiting activation of IRF3 [35, 81]. Deletion **NS1** dramatically attenuates influenza viruses [35, 81]. Inhibition of RLR signaling is a critical event in the lifecycle of many viral pathogens, for example, influenza virus [82, 83]. Influenza NS1 protein inhibits RIG-I signaling to NF-κB and IRF3 by inhibiting the E3 ubiquitin ligase TRIM 25 required for its function [33]. Furthermore, influenza virus NS1 binds to dsRNA that would trigger the RIG-I/MDA5 antiviral response. Finally, the influenza virus polymerase activity depends upon cellular mRNA, thereby depleting host mRNAs, which has been postulated to inhibit host antiviral gene expression [45]. Similar to herpesviruses, influenza virus undergoes replication in the nucleus minimizing detection by intracytosolic nucleic acid sensors dsRNA binding by the influenza. Thus, influenza virus appears to encode inhibitors of nucleic acid sensing, host antiviral gene expression, interferon response, dsRNA, and physical separation of signal transduction components from the innate immune sensors. These mechanisms are a recurrent theme in viral innate immune evasion.

Retroviruses are highly successful at evading innate and adaptive immune responses. The rapid evolution of HIV envelope proteins and their heavy glycosylation results in epitopes that are not conducive to an adaptive immune response. The well-studied mechanisms for retroviral immune evasion include the infection and apoptotic destruction of HIV-1- or HIV-2-infected T cells. It is interesting to note that HIV protease degrades human bcl-2 [84] and that HIV Nef, an HIV accessory' protein, induces apoptosis [85]. Thus this mechanism of HIV induction of apoptosis breaks the typical paradigm where viruses encode proteins that inhibit apoptosis (see Table 1).

HIV encodes several other "accessory" proteins that are essential for HIV infectivity and pathogenesis *in vivo*. These proteins antagonize the innate immune response in several ways. Nef mediates activation of MAPK signaling to AP-1, which is suggested to activate viral replication [86, 87]. HIV **Vif** and **Vpr** degrade IRF3, thereby inhibiting signaling to IRF3 and interferon production [88]. Thus, although inhibition of adaptive immunity by HIV is well known, innate immune evasion plays an important role in HIV pathogenesis.

5. Conclusion: Innate Immunoevasion—From Insight to Innovation

Many viral mechanisms have evolved to evade the immune response. Surprisingly, the general outline of innate antiviral mechanism is remarkably persistent throughout evolution, such as DNA restriction/dicing and programmed cell death; however, differences between innate immune responses of distinct organisms are often more striking and may hint at novel innate immune evasion pathways still undiscovered in mammalian virus-host interactions. Signaling to interferon resembles antiviral protein induction in *Drosophila* and in some respects, even in bacteria. The evolutionary conservation of these mechanisms suggests their study will advance understanding of viral pathogenesis and that these pathways would be worthy targets of antiviral inhibitors.

In this regard, there are several promising antiviral therapies targeting viral innate immunoevasion genes. HCV protease inhibitors have been suggested to inhibit HCV innate immunoevasion, presumably by preventing MAVS digestion [38, 89], and thereby permitting critical signaling to interferon. An *in vitro* study of the N1 vaccinia virus virulence factor and innate immune evasion protein identified chemical inhibitors of its antiapoptotic function [90]. This is surprising as N1 does not mediate cell death *in vitro*, where these inhibitors were tested [91, 92]. These findings highlight the difficulty of studying certain innate immune inhibitors *in vitro*. Nevertheless, targeting potent virulence factors of viral pathogens represent a promising and entirely new approach to antiviral drug design—beyond drugs that exclusively target viral enzymes responsible for replication. Perhaps the most promising application of these studies is in the development of highly immunogenic live vaccines that contain deletions of innate immune evasion genes outlined here. Such vaccine would have the potential to be safer and potentially more immunogenic vaccine viruses by virtue of their attenuated ability to mediate innate immune suppression.

Abbreviations

PAMP: Pathogen-associated molecular pattern
NLR: Nucleotide oligomerization domain-Like receptor
TLR: Toll-like receptor
RLR: RIG-I-like receptor
RIG-I: Retinoic acid-inducible gene-I
MDA-5: Melanoma differentiation-associated gene-5
CARD: Caspase activation and recruitment domain
MAVS: Mitochondrial antiviral signaling protein
LPS: Lipopolysaccharide
TIR: Toll/IL-1 interacting receptor
NLRP3: Nucleotide oligomerization domain-like receptor Protein 3
ASC: Apoptosis-associated speck-like protein containing caspase recruitment domain
DAI: DNA-dependent activator of interferon
STING: Stimulator of interferon genes
CRISPR: Clustered regularly sPaced short palindromic repeats
Psp: Phage shock protein
VACV: Vaccinia virus
EBV: Epstein-Barr virus
KSHV: Kaposi'ssarcoma herpesvirus

HCV: Hepatitis C virus
NPH-I: Poxvirus nucleoside triphosphate phosphohydrolase I
IFN: Interferon
NS1: Nonstructural protein 1
DDX3: DEAD/H Box 3
IKK: Inhibitor ofkappa-B kinase
TBK: TANK-binding kinase 1
IRAK: IL-1 receptor-associated kinase-1
IRF3: Interferon response factor 3
IL: Interleukin
AIM-2: Absent in melanoma-2
TRIM 25: Tripartite motif-containing protein 25.

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References

[1] C. A. Janeway Jr., "Approaching the asymptote? Evolution and revolution in immunology," *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 54, no. 1, pp. 1–13, 1989.

[2] M. Yoneyama, M. Kikuchi, T. Natsukawa et al., "The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses," *Nature Immunology*, vol. 5, no. 7, pp. 730–737, 2004.

[3] T. Kawai, K. Takahashi, S. Sato et al., "IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction," *Nature Immunology*, vol. 6, no. 10, pp. 981–988, 2005.

[4] M. Yoneyama, M. Kikuchi, K. Matsumoto et al., "Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity," *Journal of Immunology*, vol. 175, no. 5, pp. 2851–2858, 2005.

[5] A. P. Kalverda, G. S. Thompson, A. Vogel et al., "Poxvirus K7 protein adopts a Bcl-2 fold: biochemical mapping of its interactions with human DEAD box RNA helicase DDX3," *Journal of Molecular Biology*, vol. 385, no. 3, pp. 843–853, 2009.

[6] N. K. Tanner and P. Linder, "DExD/H box RNA helicases: from generic motors to specific dissociation functions," *Molecular Cell*, vol. 8, no. 2, pp. 251–262, 2001.

[7] S. Rothenfusser, N. Goutagny, G. DiPerna et al., "The RNA helicase Lgp2 inhibits TLR-independent sensing of viral replication by retinoic acid-inducible gene-I," *Journal of Immunology*, vol. 175, no. 8, pp. 5260–5268, 2005.

[8] T. Satoh, H. Kato, Y. Kumagai et al., "LGP2 is a positive regulator of RIG-I- and MDA5-mediated antiviral responses," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 4, pp. 1512–1517, 2010.

[9] R. B. Seth, L. Sun, C. K. Ea, and Z. J. Chen, "Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-κB and IRF3," *Cell*, vol. 122, no. 5, pp. 669–682, 2005.

[10] Q. Sun, L. Sun, H. H. Liu et al., "The specific and essential role of MAVS in antiviral innate immune responses," *Immunity*, vol. 24, no. 5, pp. 633–642, 2006.

[11] S. Akira, "Mammalian Toll-like receptors," *Current Opinion in Immunology*, vol. 15, no. 2, p. 238, 2003.

[12] F. Heil, H. Hemmi, H. Hochrein et al., "Species-specific recognition of single-stranded RNA via Till-like receptor 7 and 8," *Science*, vol. 303, no. 5663, pp. 1526–1529, 2004.

[13] S. Sharma, B. R. TenOever, N. Grandvaux, G. P. Zhou, R. Lin, and J. Hiscott, "Triggering the interferon antiviral response through an IKK-related pathway," *Science*, vol. 300, no. 5622, pp. 1148–1151, 2003.

[14] N. Silverman and T. Maniatis, "NF-κB signaling pathways in mammalian and insect innate immunity," *Genes and Development*, vol. 15, no. 18, pp. 2321–2342, 2001.

[15] S. M. McWhirter, K. A. Fitzgerald, J. Rosains, D. C. Rowe, D. T. Golenbock, and T. Maniatis, "IFN-regulatory factor 3-dependent gene expression is defective in Tbk1-deficient mouse embryonic fibroblasts," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 1, pp. 233–238, 2004.

[16] S. E. Doyle, S. A. Vaidya, R. O'Connell et al., "IRF3 mediates a TLR3/TLR4-specific antiviral gene program," *Immunity*, vol. 17, no. 3, pp. 251–263, 2002.

[17] G. Gautier, M. Humbert, F. Deauvieau et al., "A type I interferon autocrine-paracrine loop is involved in Toll-like receptor-induced interleukin-12p70 secretion by dendritic cells," *Journal of Experimental Medicine*, vol. 201, no. 9, pp. 1435–1446, 2005.

[18] M. M. Whitmore, M. J. DeVeer, A. Edling et al., "Synergistic activation of innate immunity by double-stranded RNA and CpG DNA promotes eahanced antitumor activity," *Cancer Research*, vol. 64, no. 16, pp. 5850–5860, 2004.

[19] K. Hoebe, X. Du, P. Georgel et al., "Identification of Lps2 as a key transducer of MyD88-independent TIR signalling," *Nature*, vol. 424, no. 6950, pp. 743–748, 2003.

[20] L. Franchi, T. Eigenbrod, R. Muñoz-Planillo, and G. Nuñez, "The inflammasome: a caspase-1-activation platform that regulates immune responses and disease pathogenesis," *Nature Immunology*, vol. 10, no. 3, pp. 241–247, 2009.

[21] V. Hornung, A. Ablasser, M. Charrel-Dennis et al., "AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC," *Nature*, vol. 458, no. 7237, pp. 514–518, 2009.

[22] M. Lamkanfi and V. M. Dixit, "Inflammasomes: guardians of cytosolic sanctity," *Immunological Reviews*, vol. 227, no. 1, pp. 95–105, 2009.

[23] K. J. Ishii, C. Coban, H. Kato et al., "A toll-like receptor-independent antiviral response induced by double-stranded B-form DNA," *Nature Immunology*, vol. 7, no. 1, pp. 40–48, 2006.

[24] D. B. Stetson and R. Medzhitov, "Recognition of cytosolic DNA activates an IRF3-dependent innate immune response," *Immunity*, vol. 24, no. 1, pp. 93–103, 2006.

[25] Z. Wang, M. K. Choi, T. Ban et al., "Regulation of innate immune responses by DAI (DLM-1/ZBP1) and other DNA-sensing molecules," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 14, pp. 5477–5482, 2008.

[26] D. Bikard and L. A. Marraffini, "Innate and adaptive immunity in bacteria: mechanisms of programmed genetic variation to fight bacteriophages," *Current Opinion in Immunology*, vol. 24, no. 1, pp. 15–20, 2012.

[27] W. C. Cheng and J. M. Hardwick, "A quorum on bacterial programmed cell death," *Molecular Cell*, vol. 28, no. 4, pp. 515–517, 2007.

[28] Y.-H. Han, Y.-J. Luo, Q. Wu et al., "RNA-based immunity terminates viral infection in adult Drosophila in the absence of viral suppression of RNA interference: characterization of viral small interfering RNA populations in wild-type and mutant flies," *Journal of Virology*, vol. 85, no. 24, pp. 13153–13163, 2011.

[29] J. H. Wang, S. Valanne, and M. Ramet, "Drosophila as a model for antiviral immunity," *World Journal of Biological Chemistry*, vol. 1, no. 5, pp. 151–159, 2010.

[30] D. Galiana-Arnoux, C. Dostert, A. Schneemann, J. A. Hoffmann, and J. L. Imler, "Essential function in vivo for Dicer-2 in host defense against RNA viruses in drosophila," *Nature Immunology*, vol. 7, no. 6, pp. 590–597, 2006.

[31] N. E. Crook, R. J. Clem, and L. K. Miller, "An apoptosis-inhibiting baculovirus gene with a zinc finger-like motif," *Journal of Virology*, vol. 67, no. 4, pp. 2168–2174, 1993.

[32] B. A. Hay, T. Wolff, and G. M. Rubin, "Expression of baculovirus P35 prevents cell death in Drosophila," *Development*, vol. 120, no. 8, pp. 2121–2129, 1994.

[33] R. L. Kuo, C. Zhao, M. Malur, and R. M. Krug, "Influenza A virus strains that circulate in humans differ in the ability of their NS1 proteins to block the activation of IRF3 and interferon-β transcription," *Virology*, vol. 408, no. 2, pp. 146–158, 2010.

[34] J. O. Langland and B. L. Jacobs, "The role of the PKR-inhibitory genes, E3L and K3L, in determining vaccinia virus host range," *Virology*, vol. 299, no. 1, pp. 133–141, 2002.

[35] G. DiPerna, J. Stack, A. G. Bowie et al., "Poxvirus protein N1L targets the I-κB kinase complex, inhibits signaling to NF-κB by the tumor necrosis factor superfamily of receptors, and inhibits NF-κB and IRF3 signaling by toll-like receptors," *The Journal of Biological Chemistry*, vol. 279, no. 35, pp. 36570–36578, 2004.

[36] A. Bowie, E. Kiss-Toth, J. A. Symons, G. L. Smith, S. K. Dower, and L. A. J. O'Neill, "A46R and A52R from vaccinia virus are antagonists of host IL-1 and toll-like receptor signaling," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 18, pp. 10162–10167, 2000.

[37] J. M. Gonzlez and M. Esteban, "A poxvirus Bcl-2-like gene family involved in regulation of host immune response: sequence similarity and evolutionary history," *Virology Journal*, vol. 7, article 59, 2010.

[38] C. L. Johnson, D. M. Owen, and M. Gale Jr., "Functional and therapeutic analysis of hepatitis C virus NS3·4A protease control of antiviral immune defense," *The Journal of Biological Chemistry*, vol. 282, no. 14, pp. 10792–10803, 2007.

[39] A. Hosui, K. Ohkawa, H. Ishida et al., "Hepatitis C virus core protein differently regulates the JAK-STAT signaling pathway under interleukin-6 and interferon-γ stimuli," *The Journal of Biological Chemistry*, vol. 278, no. 31, pp. 28562–28571, 2003.

[40] M. J. Gale Jr., M. J. Korth, and M. G. Katze, "Repression of the PKR protein kinase by the hepatitis C virus NS5A protein: a potential mechanism of interferon resistance," *Clinical and Diagnostic Virology*, vol. 10, no. 2-3, pp. 157–162, 1998.

[41] B. Moss, "Poxviridae: the viruses and their replication," in *Fields Virology*, D. M. Knipe and P. M. Howley, Eds., vol. 2, pp. 2849–2883, Lippincott, Williams & Wilkins, Philadelphia, Pa, USA, 2001.

[42] K. Li, E. Foy, J. C. Ferreon et al., "Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 8, pp. 2992–2997, 2005.

[43] J. P. Parisien, D. Bamming, A. Komuro et al., "A shared interface mediates Paramyxovirus interference with antiviral RNA helicases MDA5 and LGP2," *Journal of Virology*, vol. 83, no. 14, pp. 7252–7260, 2009.

[44] S. M. Gregory, B. K. Davis, J. A. West et al., "Discovery of a viral NLR homolog that inhibits the inflammasome," *Science*, vol. 331, no. 6015, pp. 330–334, 2011.

[45] M. M. Rahman and G. McFadden, "Myxoma virus lacking the pyrin-like protein M013 is sensed in human myeloid cells by both NLRP3 and multiple toll-like receptors, which independently activate the inflammasome and NF-κB innate response pathways," *Journal of Virology*, vol. 85, no. 23, pp. 12505–12517, 2011.

[46] M. Thome, P. Schneider, K. Hofmann et al., "Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors," *Nature*, vol. 386, no. 6624, pp. 517–521, 1997.

[47] S. Henderson, D. Huen, M. Rowe, C. Dawson, G. Johnson, and A. Rickinson, "Epstein-Barr virus-coded BHRF1 protein, a viral homologue of Bcl-2, protects human B cells from programmed cell death," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 90, no. 18, pp. 8479–8483, 1993.

[48] W. L. Marshall, C. Yim, E. Gustafson et al., "Epstein-Barr virus encodes a novel homolog of the bcl-2 oncogene that inhibits apoptosis and associates with Bax and Bak," *Journal of Virology*, vol. 73, no. 6, pp. 5181–5185, 1999.

[49] R. Sarid, T. Sato, R. A. Bohenzky, J. J. Russo, and Y. Chang, "Kaposi's sarcoma-associated herpesvirus encodes a functional Bcl-2 homologue," *Nature Medicine*, vol. 3, no. 3, pp. 293–298, 1997.

[50] T. W. Sedlak, Z. N. Oltvai, E. Yang et al., "Multiple Bcl-2 family members demonstrate selective dimerizations with Bax," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 17, pp. 7834–7838, 1995.

[51] D. C. S. Huang, J. M. Adams, and S. Cory, "The conserved N-terminal BH4 domain of Bcl-2 homologues is essential for inhibition of apoptosis and interaction with CED-4," *EMBO Journal*, vol. 17, no. 4, pp. 1029–1039, 1998.

[52] A. Gross, J. M. McDonnell, and S. J. Korsmeyer, "BCL-2 family members and the mitochondria in apoptosis," *Genes and Development*, vol. 13, no. 15, pp. 1899–1911, 1999.

[53] M. Mandal, S. B. Maggirwar, N. Sharma, S. H. Kaufmann, S. C. Sun, and R. Kumar, "Bcl-2 prevents CD95 (Fas/APO-1)-induced degradation of lamin B and poly(ADP-ribose) polymerase and restores the NF-κB signaling pathway," *The Journal of Biological Chemistry*, vol. 271, no. 48, pp. 30354–30359, 1996.

[54] L. A. Kirshenbaum, "Bcl-2 intersects the NFκB signalling pathway and suppresses apoptosis in ventricular myocytes," *Clinical and Investigative Medicine*, vol. 23, no. 5, pp. 322–330, 2000.

[55] K. M. Regula, K. Ens, and L. A. Kirshenbaum, "IKKβ is required for Bcl-2-mediated NF-κB activation in ventricular myocytes," *The Journal of Biological Chemistry*, vol. 277, no. 41, pp. 38676–38682, 2002.

[56] Q. Li, G. Estepa, S. Memet, A. Israel, and I. M. Verma, "Complete lack of NF-κB activity in IKK1 and IKK2 double-deficient mice: additional defect in neurulation," *Genes and Development*, vol. 14, no. 14, pp. 1729–1733, 2000.

[57] M. Karin and Y. Ben-Neriah, "Phosphorylation meets ubiquitination: the control of NF-κB activity," *Annual Review of Immunology*, vol. 18, pp. 621–663, 2000.

[58] J. V. Hartig and K. Förstemann, "Loqs-PD and R2D2 define independent pathways for RISC generation in Drosophila," *Nucleic Acids Research*, vol. 39, no. 9, pp. 3836–3851, 2011.

[59] M. Hussain, R. J. Taft, and S. Asgari, "An insect virus-encoded microrna regulates viral replication," *Journal of Virology*, vol. 82, no. 18, pp. 9164–9170, 2008.

[60] G. Jovanovic, C. Engl, and M. Buck, "Physical, functional and conditional interactions between ArcAB and phage shock proteins upon secretin-induced stress in *Escherichia coli*," *Molecular Microbiology*, vol. 74, no. 1, pp. 16–28, 2009.

[61] R. A. Zambon, M. Nandakumar, V. W. Vakharia, and L. P. Wu, "The Toll pathway is important for an antiviral response in Drosophila," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 20, pp. 7257–7262, 2005.

[62] S. Deddouche, N. Matt, A. Budd et al., "The DExD/H-box helicase Dicer-2 mediates the induction of antiviral activity in drosophila," *Nature Immunology*, vol. 9, no. 12, pp. 1425–1432, 2008.

[63] M. E. Grether, J. M. Abrams, J. Agapite, K. White, and H. Steller, "The head involution defective gene of Drosophila melanogaster functions in programmed cell death," *Genes and Development*, vol. 9, no. 14, pp. 1694–1708, 1995.

[64] E. C. LaCasse, S. Baird, R. G. Korneluk, and A. E. MacKenzie, "The inhibitors of apoptosis (IAPs) and their emerging role in cancer," *Oncogene*, vol. 17, no. 25, pp. 3247–3259, 1998.

[65] A. Alcamí, J. A. Symons, and G. L. Smith, "The vaccinia virus soluble alpha/beta interferon (IFN) receptor binds to the cell surface and protects cells from the antiviral effects of IFN," *Journal of Virology*, vol. 74, no. 23, pp. 11230–11239, 2000.

[66] B. Billings, S. A. Smith, Z. Zhang, D. K. Lahiri, and G. J. Kotwal, "Lack of N1L gene expression results in a significant decrease of vaccinia virus replication in mouse brain," *Annals of the New York Academy of Sciences*, vol. 1030, pp. 297–302, 2004.

[67] Z. Zhang, M. R. Abrahams, L. A. Hunt et al., "The vaccinia virus N1L protein influences cytokine secretion in vitro after infection," *Annals of the New York Academy of Sciences*, vol. 1056, pp. 69–86, 2005.

[68] M. T. Harte, I. R. Haga, G. Maloney et al., "The poxvirus protein A52R targets toll-like receptor signaling complexes to suppress host defense," *Journal of Experimental Medicine*, vol. 197, no. 3, pp. 343–351, 2003.

[69] C. A. Ray, R. A. Black, S. R. Kronheim et al., "Viral inhibition of inflammation: cowpox virus encodes an inhibitor of the interleukin-1β converting enzyme," *Cell*, vol. 69, no. 4, pp. 597–604, 1992.

[70] R. Mills, M. Rozanov, A. Lomsadze, T. Tatusova, and M. Borodovsky, "Improving gene annotation of complete viral genomes," *Nucleic Acids Research*, vol. 31, no. 23, pp. 7041–7055, 2003.

[71] Q. Huang, A. M. Petros, H. W. Virgin, S. W. Fesik, and E. T. Olejniczak, "Solution structure of the BHRF1 protein from Epstein-Barr virus, a homolog of human Bcl-2," *Journal of Molecular Biology*, vol. 332, no. 5, pp. 1123–1130, 2003.

[72] M. Altmann and W. Hammerschmidt, "Epstein-Barr virus provides a new paradigm: a requirement for the immediate inhibition of apoptosis," *PLoS Biology*, vol. 3, no. 12, article e404, 2005.

[73] N. J. Bump, M. Hackett, M. Hugunin et al., "Inhibition of ICE family proteases by baculovirus antiapoptotic protein p35," *Science*, vol. 269, no. 5232, pp. 1885–1888, 1995.

[74] E. Meinl, H. Fickenscher, M. Thome, J. Tschopp, and B. Fleckenstein, "Anti-apoptotic strategies of lymphotropic viruses," *Immunology Today*, vol. 19, no. 10, pp. 474–479, 1998.

[75] T. Kondo, T. Yokokura, and S. Nagata, "Activation of distinct caspase-like proteases by Fas and reaper in Drosophila cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 22, pp. 11951–11956, 1997.

[76] N. Field, W. Low, M. Daniels et al., "KSHV vFLIP binds to IKK-γ to activate IKK," *Journal of Cell Science*, vol. 116, no. 18, pp. 3721–3728, 2003.

[77] M. Diaz-Guerra, J. S. Kahn, and M. Esteban, "A mutation of the nucleoside triphosphate phosphohydrolase I (NPH-I) gene confers sensitivity of vaccinia virus to interferon," *Virology*, vol. 197, no. 1, pp. 485–491, 1993.

[78] A. J. V. Thompson and J. G. McHutchison, "Antiviral resistance and specifically targeted therapy for HCV (STAT-C)," *Journal of Viral Hepatitis*, vol. 16, no. 6, pp. 377–387, 2009.

[79] H. Chung, T. Watanabe, M. Kudo, and T. Chiba, "Hepatitis C virus core protein induces homotolerance and cross-tolerance to Toll-like receptor ligands by activation of Toll-like receptor 2," *Journal of Infectious Diseases*, vol. 202, no. 6, pp. 853–861, 2010.

[80] W. Lin, W. H. Choe, Y. Hiasa et al., "Hepatitis C virus expression suppresses interferon signaling by degrading STAT1," *Gastroenterology*, vol. 128, no. 4, pp. 1034–1041, 2005.

[81] A. L. Hartman, J. S. Towner, and S. T. Nichol, "A C-terminal basic amino acid motif of Zaire ebolavirus VP35 is essential for type I interferon antagonism and displays high identity with the RNA-binding domain of another interferon antagonist, the NS1 protein of influenza a virus," *Virology*, vol. 328, no. 2, pp. 177–184, 2004.

[82] A. Pichlmair, O. Schulz, C. P. Tan et al., "RIG-I-mediated antiviral responses to single-stranded RNA bearing 5′-phosphates," *Science*, vol. 314, no. 5801, pp. 997–1001, 2006.

[83] Y. Watanabe, N. Ohtaki, Y. Hayashi, K. Ikuta, and K. Tomonaga, "Autogenous translational regulation of the borna disease virus negative control factor X from polycistronic mRNA using host RNA helicases," *PLoS Pathogens*, vol. 5, no. 11, Article ID e1000654, 2009.

[84] P. R. Strack, M. W. Frey, C. J. Rizzo et al., "Apoptosis mediated by HIV protease is preceded by cleavage of bcl-2," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 18, pp. 9571–9576, 1996.

[85] A. Rasola, D. Gramaglia, C. Boccaccio, and P. M. Comoglio, "Apoptosis enhancement by the HIV-1 Nef protein," *Journal of Immunology*, vol. 166, no. 1, pp. 81–88, 2001.

[86] L. Tuosto, B. Marinari, M. Andreotti, M. Federico, and E. Piccolella, "Vav exchange factor counteracts the HIV-1 Nef-mediated decrease of plasma membrane GM1 and NF-AT activity in T cells," *European Journal of Immunology*, vol. 33, no. 8, pp. 2186–2196, 2003.

[87] A. Varin, S. K. Manna, V. Quivy et al., "Exogenous Nef protein activates NF-κB, AP-1, and c-Jun N-terminal kinase and stimulates HIV transcription in promonocytic cells: role in AIDS pathogenesis," *The Journal of Biological Chemistry*, vol. 278, no. 4, pp. 2219–2227, 2003.

[88] A. Okumura, T. Alce, B. Lubyova, H. Ezelle, K. Strebel, and P. M. Pitha, "HIV-1 accessory proteins VPR and Vif modulate antiviral response by targeting IRF-3 for degradation," *Virology*, vol. 373, no. 1, pp. 85–97, 2008.

[89] J. A. Thomson and R. B. Perni, "Hepatitis C virus NS3·4A protease inhibitors: countering viral subversion in vitro and

showing promise in the clinic," *Current Opinion in Drug Discovery and Development*, vol. 9, no. 5, pp. 606–617, 2006.

[90] N. Bartlett, J. A. Symons, D. C. Tscharke, and G. L. Smith, "The vaccinia virus N1L protein is an intracellular homodimer that promotes virulence," *Journal of General Virology*, vol. 83, no. 8, pp. 1965–1976, 2002.

[91] G. J. Kotwal, A. W. Hugin, and B. Moss, "Mapping and insertional mutagenesis of a vaccinia virus gene encoding a 13,800-Da secreted protein," *Virology*, vol. 171, no. 2, pp. 579–587, 1989.

[92] A. V. Cheltsov, M. Aoyagi, A. Aleshin et al., "Vaccinia virus virulence factor N1L is a novel promising target for antiviral therapeutic intervention," *Journal of Medicinal Chemistry*, vol. 53, no. 10, pp. 3899–3906, 2010.

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Research Article

Association of Active Human Herpesvirus-6, -7 and Parvovirus B19 Infection with Clinical Outcomes in Patients with Myalgic Encephalomyelitis/Chronic Fatigue Syndrome

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Frequency of active human herpesvirus-6, -7 (HHV-6, HHV-7) and parvovirus B19 (B19) infection/coinfection and its association with clinical course of ME/CFS was evaluated. 108 ME/CFS patients and 90 practically healthy persons were enrolled in the study. Viral genomic sequences were detected by PCR, virus-specific antibodies and cytokine levels—by ELISA, HHV-6 variants—by restriction analysis. Active viral infection including concurrent infection was found in 64.8% (70/108) of patients and in 13.3% (12/90) of practically healthy persons. Increase in peripheral blood leukocyte DNA HHV-6 load as well as in proinflammatory cytokines' levels was detected in patients during active viral infection. Definite relationship was observed between active betaherpesvirus infection and subfebrility, lymphadenopathy and malaise after exertion, and between active B19 infection and multijoint pain. Neuropsychological disturbances were detected in all patients. The manifestation of symptoms was of more frequent occurrence in patients with concurrent infection. The high rate of active HHV-6, HHV-7 and B19 infection/coinfection with the simultaneous increase in plasma proinflammatory cytokines' level as well as the association between active viral infection and distinctive types of clinical symptoms shows necessity of simultaneous study of these viral infections for identification of possible subsets of ME/CFS.

1. Introduction

Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is a disease characterized by profound disabling fatigue lasting at least 6 months and accompanied by a combination of nonspecific symptoms. According to the 1994 US Center's for Disease Control and Prevention (CDC) case definition, which at present, is widespread in research and clinical practice, at least four out of eight symptoms (impaired memory or concentration, sore throat, tender cervical or axillary lymph nodes, muscle pain, multi-joint pain, new headaches, sleep disturbances and post-exertion malaise) should be present in cases of ME/CFS [1]. During the clinical course of disease multiple body systems are affected by immune, neuroendocrine, musculoskeletal as well as psychiatric factors that reflect on the heterogeneity of the disease. Because fatigue is a common symptom of many diseases, a wide differential diagnosis needs to be done. The observation that many cases of the disease begin with a flu-like illness has prompted the hypothesis that viral infections are implicated in this disorder.

Infections of human β-herpesviruses-human herpesvirus-6 and -7 (HHV-6, HHV-7), cytomegalovirus (CMV) [2–4], Epstein-Barr virus [5, 6], parvovirus B19 (B19) [7–9], and enterovirus [10] are suggested as etiological agents for ME/CFS. The major hypothesis of the pathogenesis of the disease is that persistent viral infections may trigger and lead to chronic activation of the immune system with abnormal

regulation of cytokine production [11]. However, until now the role of viral infections as etiological agents for ME/CFS has been evaluated inconsistently [12–15]. Identification of specific biomarkers for differential diagnosis of ME/CFS has intensively been studied [16–19].

HHV-6 and HHV-7 are lymphotropic, neurotropic, and immunomodulating viruses which primary infection is followed by lifelong persistency. Reactivation of viruses can provoke the development of abnormalities involving the immune system and nervous system and probably may trigger ME/CFS [14]. Two distinct variants of HHV-6 (A and B) have been described [19] and the human glycoprotein CD46 has been recorded as a receptor molecule for both variants [20]. Clinical features of HHV-6A infection remain unclear.

Human parvovirus B19 was first discovered by Cossart et al. [21] in the sera of healthy blood donors. The virus is ubiquitous and course of infection depends on the host's hematological status and immune response. Cellular receptor for B19 is globoside (blood group P antigen) [22]. Tissue distribution of P antigen may explain the clinical manifestation of the viral infection [23].

The appearance of antibodies to B19 is associated with clearance of the virus from the bloodstream; however, the high frequency of persistence of B19 DNA in tissue of healthy persons under the presence of anti-B19 antibodies indicates that complete eradication of the virus from host body has not occurred [24]. It is unclear how often ME/CFS appears after B19 infection and whether B19 viremia or other factors cause ME/CFS [9].

The aim of this study was to determine frequency of active HHV-6, HHV-7, and B19 infection/coinfection and to evaluate association of active single and concurrent infection with clinical outcomes in cases of ME/CFS.

2. Materials and Methods

2.1. Patients. One hundred eight randomly selected patients (66 females, 42 males; mean age 37 years) with clinically diagnosed ME/CFS after rigorous examination of criteria (fatigue for at least six months and at least four of eight symptoms: postexertional malaise, impaired memory or concentration, unrefreshing sleep, muscle pain, multijoint pain, tender cervical or axillary lymph nodes, sore throat, headache) corresponding to the 1994 CDC definition and 90 practically healthy persons (55 females, 35 males; mean age 39 years) were investigated for evidence of HHV-6, HHV-7 and B19 infection/coinfection. The presence and frequency of clinical features in ME/CFS patients were examined in relation to active viral infection. Severity of fatigue was evaluated by Fatigue Severity Scale (FSS) with maximal score of 72 points [25]. The cohort was established with the approval of the Ethics Committee of the Riga Stradins University and all participants gave their informed consent prior to the examination. Ninety practically healthy persons (55 females, 35 males; mean age 39 years) were included in this examination as a control group.

2.2. HHV-6 and B19 Serology. Plasma samples were tested by ELISA kits (Panbio, Sinnamon Park, OLD, Australia) for specific anti-HHV-6 IgM and IgG class antibodies according manufacturer's protocols. Tests for antibodies against B19 were carried out using B19 IgG and IgM the anti-VP2 enzymatic immunoassay kits (Biotrin, Dublin, Ireland) in accordance with the manufacturer's recommendations.

2.3. Nested Polymerase Chain Reaction (nPCR). The technique of nPCR was used to detect viral genomic sequences in DNA isolated from peripheral blood leukocytes (PBLs) and cell free plasma (markers of persistent and active infection, resp.). Total DNA was isolated from 0.5 ml of fresh whole blood by phenol-chloroform extraction. The QIAamp Blood Kit (QIAGEN, Hilden, Germany) was used to purify DNA from 200 μL of cell-free blood plasma. The plasma samples were treated with DNase I (Fermentas, Vilnius, Lithuania) before DNA purification. To assure the quality of the PBL DNA and to exclude contamination of plasma DNA by cellular DNA, PCR was performed with primers that recognized the globin gene. PCR amplification of viral DNA was carried out in the presence of 1 μg of PBL DNA or 10 μL of plasma DNA (which corresponded to 100 μL of plasma). HHV-6, HHV-7, and B19 DNA were detected in accordance with Secchiero et al. [26], Berneman et al. [27], and Cavallo et al. [28], respectively. Positive controls (HHV-6 and HHV-7 genomic DNA; Advanced Biotechnologies Inc, Columbia, MD, USA and B19 genomic DNA isolated from viremic serum kindly provided by Prof. K.Hedman, Department of Virology, Heartman Institute, University of Helsinki) and negative controls (DNA obtained from practically healthy HHV-6, HHV-7 and B19 negative blood donor and no template DNA) were included in each experiment.

Criteria to define persistent viral infection were the presence of virus-specific IgG class antibodies in blood plasma and viral genomic sequences in DNA isolated from the whole blood. The presence of HHV-6-specific IgM class antibodies in blood plasma and viral genomic sequence in plasma DNA samples, as well as elevated titer of virus-specific IgG class antibodies, without IgM antibodies and viral genomic sequence in plasma DNA samples was defined as active HHV-6 infection. The cases with HHV-7-specific sequence in DNA isolated from cell free blood plasma were defined as active HHV-7 infection cases. The presence of HHV-7-specific antibodies was not examined due to the lack of commercial test systems for IgM class antibody detection. The cases with persistent infection and without markers of active viral infection were defined as latent stage of persistent infection (latent infection) cases. B19 genomic sequence in plasma DNA samples with or without the presence of IgM class specific antibodies and the presence of viral sequence in PBL DNA samples together with IgM class specific antibodies in blood plasma we defined as active B19 infection.

2.4. Quantitative Real-Time PCR. The viral load of HHV-6 in PBL DNA samples from patients with latent and active viral infections was determined using the HHV-6 Real-Time Alert Q-PCR kit (Nanogen Advanced Diagnostics,

Buttigliera Alta, Italy) and an Applied Biosystems 7500 Real-time PCR System (Applied Biosystems, Carlsbad, CA, USA), in accordance with the manufacturer's recommendations.

2.5. Restriction Endonuclease Analysis. Restriction endonuclease analysis was carried out using the restriction enzyme *Hind*III (Fermentas, Vilnius, Lithuania), which cleaves the 163 bp HHV-6B amplimer into two fragments of 66 and 97 bp but does not cleave HHV-6A amplimer.

2.6. Assay for Cytokine Determination. Endogen Human ELISA kits (Pierce Biotechnology, Rockford, IL, USA) were used to detect the level of tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-4 in plasma samples from ME/CFS patients according to the manufacturer's recommendations. The sensitivity of the ELISAs was 2 pg/mL for TNF-α and <1 pg/mL for IL-6, and <4 pg/mL for IL-4. All samples were tested in duplicate. Plasma samples were processed immediately after collection and then stored at -70°C.

2.7. Statistical Analysis. Statistical difference in the frequency of active HHV-6, HHV-7, and B19 infection/coinfection between tested groups was assessed by Odds ratio, 95% CI values and Fisher's exact test and in the levels of cytokines-by Student's t-test, using MedCalc software for Windows, version 12.2.1; a value of $P < 0.05$ was considered to be significant.

3. Results

3.1. HHV-6 and B-19 Serology. Specific anti-HHV-6 antibodies were detected in 87/108 (80.6%) plasma samples (IgG-71, IgM-3, IgM + IgG-13) from the ME/CFS patients versus 69/90 (76.7%) practically healthy persons' plasma samples (IgG-67, IgM + IgG-2) and specific anti-B19 antibodies in 92/108 (85.2%) plasma samples (IgG-62, IgM-6, IgM + IgG-24) from the ME/CFS patients versus 55/90 (61.1%) plasma samples (IgG-44, IgM-2, IgM + IgG-9) from the practically healthy persons.

3.2. Prevalence of Active HHV-6, HHV-7, and B19 Infections. Active viral infection/coinfection was detected in 70 (64.8%) and latent—in 32 (29.6%) out of 108 patients with ME/CFS. Six (5.6%) patients were negative for viral infection (Table 1). The rate of active viral infection was significantly higher in patients comparing to the rate in the practically healthy persons (70/108 and 12/90, resp.; Odds ratio 0.14, 95% CI 0.07–0.26, $P = 0.0001$). In the patients significant difference was detected between the frequency of single active and concurrent active viral infection (41/70, 58.6% and 29/70, 41.4%, resp.; Odds ratio 2.0, 95% CI 1.02–3.92, $P = 0.044$). Among the patients who were infected with a single virus, the rate of HHV-7 active infection (40%) was significantly higher in comparison with B19 (15.7%, Odds ratio 3.58, 95% CI 1.60–7.97, $P = 0.002$) and HHV-6 active infection (2.9%, Odds ratio 22.7, 95% CI 5.13–100.1, $P < 0.0001$). No significant difference was detected between the frequency of active concurrent dual HHV-6 + HHV-7 and dual HHV-7 + B19 infection (Odds ratio 0.61, 95% CI 0.25–1.47, $P = 0.273$).

Table 1: Frequency of Active HHV-6, HHV-7 and B19 Infection in ME/CFS Patients and Practically Health Persons.

Viral infection	Plasma samples ($n = 108$)	Practically health persons ($n = 90$)
Active viral infection	70/108	12/90
Single HHV-6	2/108	0/90
Single HHV-7	28/108	10/90
Single B19	11/108	2/90
Dual HHV-6 + HHV-7	10/108	0/90
Dual HHV-7 + B19	15/108	0/90
Triple HHV-6 + HHV-7 + B19	4/108	0/90
Latent viral infection	32/108	53/90
Without viral infection	6/108	25/90

HHV-6B was identified in 15 and HHV-6A in one out of 16 PBL and plasma DNA samples.

3.3. HHV-6 DNA Load in PBL DNA Samples . The number of HHV-6 DNA copies in PBL DNA of the ME/CFS patients with and without HHV-6 viremia was compared. A clear increase of HHV-6 load in PBL DNA was detected in 16 patients with plasma viremia in comparison with seven patients without it ($132.61 \pm 41.38 \times 10^3$ and $8.73 \pm 3.96 \times 10^3$ copies/μg DNA, resp.).

3.4. Relationship between Plasma Level of TNF-α, IL-6, and IL-4 and Active Viral Infection/Coinfection. To investigate the relationship between the active viral infections and plasma cytokine levels, the levels of proinflammatory (TNF-α, IL-6) and anti-inflammatory (IL-4) cytokines were measured in 106 ME/CFS patients (Table 2). The mean levels of TNF-α and IL-6 cytokines were significantly higher in patients with active viral infection/coinfection (52.51 ± 15.13 pg/mL, 18.59 ± 3.56 pg/mL, resp.) than in those with latent (18.81 ± 2.52 pg/mL, 2.56 ± 1.02 pg/mL, resp.; $P < 0.0001$) or without viral infections (7.71 ± 3.07 pg/mL, 1.32 ± 3.07 pg/mL, resp.; $P < 0.0001$). No significant difference was detected between expression levels of TNF-α in the patients with active single HHV-7 and active single B19 infection, as well as with dual active HHV-6 + HHV-7 and dual active HHV-7 + B19 coinfection (Table 2).

The highest level of TNF-α was detected in patients with active triple HHV-6+HHV-7 + B19 coinfection. Significantly higher level of IL-6 expression was detected in plasma samples of the patients with single active B19 infection in comparison with the patients with single active HHV-7 infection ($P < 0.001$) (Table 2). The mean levels of this cytokine were also significantly higher in patients with active concurrent HHV-7 + B19, as well as in patients with triple HHV-6 + HHV-7 + B19 infection (29.19 ± 6.26 pg/mL, $P < 0.001$) in comparison with the level in patients with dual HHV-6 + HHV-7 infection (11.22 ± 3.14 pg/mL). None of the ME/CFS patients had increased plasma level of IL-4.

Table 2: Plasma cytokine levels in patients with ME/CFS.

Viral infection	TNF-alpha (pg/mL)	IL-6 (pg/mL)	IL-4 (pg/mL)
Without ($n = 6$)	7.71 ± 3.07	1.32 ± 0.43	<2
Latent ($n = 32$)	18.81 ± 2.52	2.56 ± 1.02	<2
Active ($n = 68$) Single HHV-7 ($n = 28$)	44.81 ± 10.56	7.76 ± 2.45	<2
Single B19 ($n = 11$)	44.12 ± 11.67	15.62 ± 4.63	<2
Dual HHV-6 + HHV-7 ($n = 10$)	50.38 ± 8.74	11.22 ± 3.14	<2
Dual HHV-7 + B19 ($n = 15$)	49.89 ± 16.1	28.70 ± 3.05	<2
Triple HHV-6 + HHV-7 + B19 ($n = 4$)	73.33 ± 18.76	29.67 ± 4.52	<2

3.5. Assessment of Active Betaherpesviruses and B19 Infection/Coinfection in Association with Clinical Outcomes in ME/CFS Patients. Severe chronic fatigue for at least six months or longer was observed in all patients irrespective of the causation of the active infection (total FSS scores 58.89–60.99, $P < 0.05$). Subfebrility, tender cervical or axillary lymph nodes, and postexertional malaise were not revealed in the patients with single B19 active infection but were detected in patients with single HHV-7 active (50%, 75%, 100%, resp.), dual HHV-6 + HHV-7 (70%, 80%, 90%, resp.) as well as triple HHV-6 + HHV-7 + B19 coinfection (74.1%, 68.4%, 74.1%) (Table 3).

Although muscle pain was observed in all patients, the frequency of multijoint pain was more clearly displayed in all patients with active B19 infection, as in cases of single as well as in cases of coinfection with β-herpesviruses.

Severe postexertional malaise corresponding to "Exercise brings on my fatigue" by FSS was detected in all patients (mean score 6.94 ± 0.243 from 7 maximum) with single HHV-6, HHV-7, and in 9/10 with dual HHV-6 + HHV-7 coinfection as well as in 14/19 with triple HHV-6 + HHV-7 + B19 coinfection (90% and 74%, resp.).

Neuropsychological disturbances were observed in all 70 patients. Impaired memory was detected in 22 out of 57 (38.6%) patients with active β-herpesviruses infection/coinfection but not observed in patients with single HHV-6 and single B19 infection. Impaired concentration was detected in 34 out of 70 patients (48.6%), more frequently in patients with B19 infection. Sleep disturbances were revealed in 49 out of 70 (70%) patients, the sleepiness was more characteristic for patients with single HHV-6, B19, and dual HHV-7+B19 coinfection (2/2, 11/11 and 15/15, resp.).

Headaches of new type were observed in 16 out of 30 (53.3%) patients with B19 infection/coinfection and in 14 out of 38 (36.8%) patients with HHV-7 and dual HHV-6 + HHV-7 infection.

Chronic fatigue (for at least 6 months or longer period) was observed also in all 38 patients with latent infection and without infection (32 with latent infection and 6 without infection). Postexertional malaise (23/38, 60.5%, mean score 5.23 ± 0.135 from 7 maximum), impaired memory (34/38, 89.5%), decreased concentration (32/38, 84.2%), and sleep disturbances (24/38, 63.2%) were predominant symptoms in these patients. Subfebrility (10/38, 26.3%) and lymphadenopathy (11/38, 29%) were observed only in patients with latent single HHV-7 and dual HHV-6 + HHV-7 infection/coinfection; muscle pain (14/38, 36.8%) in patients with β-herpesviruses infection/coinfection (11/14) as well as with B19 infection/coinfection (3/14). Multijoint pain was observed in 15 out of 38 (39.5%) patients and in 10 of them B19 infection/coinfection was found. Headaches of new type were observed in 13 out of 38 (34.2%) patients. Among these 13 patients β-herpesviruses infection/coinfection was detected in 9 patients and B19 infection/coinfection in 4 patients. Clinical manifestations of the above-mentioned symptoms were not severe (total FSS scores were 42.83–48.90, $P < 0.05$).

Subfebrility, lymphadenopathy, malaise after exertion, muscle pain, multijoint pain, sleep disturbances, and headaches of new type were more frequent in patients with active viral infection/coinfection than in patients with latent infection and without infection. Whereas presence of impaired memory and impaired concentration was more frequent in patients with latent infection and without infection in comparison to the patients with active infection.

4. Discussion

ME/CFS is heterogeneous disorder with common set of symptoms that follows a viral infection. Despite efforts in the development of standardized research criteria to define ME/CFS [1, 11, 29, 30] progress in diagnosis and elucidation of the role of viral infections is slow, due to a lack of common standard clinical definition and specific biomarkers of disease.

This study is the continuation of research of HHV-6 and HHV-7 as triggering factors for ME/CFS [3]. In the study reported herein, we investigated the frequency of active HHV-6, HHV-7 and parvovirus B19 infection/coinfection and potential relationship of active viral infection with different clinical symptoms in 108 patients with clinically diagnosed ME/CFS.

The results of our study showed a high rate of HHV-6 and B19 seroprevalence among our patients and practically healthy persons. The positive rates of anti-HHV-6 and anti-B19 IgG class antibodies are similar in ME/CFS patients and practically healthy persons. At the same time, the elevated frequency of anti-HHV-6 IgM class antibodies is found in ME/CFS patients which is in concordance with previously observation by Patnaik et al. [31] and Ablashi et al. [2]. In contrast, Koelle et al. [32] and Cameron et al. [15] do not support these data. Also the elevated frequency of anti-B19 IgM class antibodies is found in ME/CFS patients in comparison with the practically healthy persons (Odds ratio 0.36, 95% CI 0.17–0.77, $P = 0.009$). High rates of active viral infection/coinfection in ME/CFS patients might be associated with insufficiency of the humoral immune response to the viruses.

We identified active viral infection in 70 (65%) ME/CFS patients, 41 of them had active single HHV-6, HHV-7, and

Table 3: Symptoms of ME/CFS in patients with active viral infection/coinfection.

Defining symptoms	Active viral infection				
	HHV-6 ($n = 2$)	HHV-7 ($n = 28$)	B19 ($n = 11$)	HHV-6 + HHV-7 ($n = 10$)	HHV-7 + B19 and HHV-6 + HHV-7 + B19 ($n = 19$)
Subfebrility	0/2	14/28	0/11	7/10	14/19
Lymphadenopathy	2/2	21/28	0/11	8/10	10/19
Malaise after exertion	2/2	28/28	0/11	9/10	14/19
Muscle pain	2/2	28/28	11/11	10/10	19/19
Multijoint pain	2/2	7/28	11/11	10/10	19/19
Impaired memory	0/2	7/28	0/11	6/10	9/19
Impaired concentration	2/2	7/28	11/11	4/10	10/19
Sleep disturbances	2/2	7/28	11/11	10/10	19/19
New headaches	0/2	14/28	11/11	0/10	5/19

B19 infection and 29-concurrent (dual HHV-6 + HHV-7, HHV-7 + B19 and triple HHV-6 + HHV-7 + B19) infection. The active single HHV-7 and B19 infection, but not active single HHV-6 or active concurrent infection, has been previously detected in Latvian blood donors [33]. The active HHV-6 infection was confirmed by the detection of viral sequence in plasma DNA samples and a concomitant increase of HHV-6 load in PBLs. This corresponds with the data demonstrated by Ihira et al. [34].

Although HHV-6A is predominant in ME/CFS patients, it was detected in only one patient that might be limited to persistency sites other than the peripheral blood [8].

Despite the fact that the precise mechanism by which betaherpesviruses and B19 impair immunological function are not completely clear, previous investigations have shown that these viruses are effective modulators of the immune response, mainly by modulating the production of proinflammatory cytokines, including TNF-α and IL-6 [35, 36]. We detected the presence of significantly higher levels of TNF-α and IL-6 in plasma samples from patients with active viral infection. This finding confirms the immunomodulating properties of these viruses and is in concordance with the data of Fletcher et al. [37]. However, our data do not corroborate with the findings of Vollmer-Conna et al. [38] that have found no significant difference of cytokine production in patients with postinfection fatigue syndrome.

The relationship between active β-herpesviruses and B19 infection/coinfection and ME/CFS clinical symptoms is not clear. In the present study, clinical features of the disease were particularly studied in detail specially in subgroups of 70 patients with different active viral infections although clinical diagnosis of ME/CFS was confirmed in the presence of four from eight analyzed symptoms [1] in all patients including those with latent viral infection and without infection. The analysis of our results showed definite relationship between active single HHV-6 and HHV-7 infection and presence of subfebrility, lymphadenopathy, and malaise after exertion, and single B19 infection with multijoint pain. By concurrent infection no clear differences in manifestations of the symptoms were detected. Neuropsychological disturbances were detected in all ME/CFS patients. Neuropsychological disturbances were detected in all ME/CFS patients.

Results of our study correspond to the newest International Consensus Criteria for clinical case definition [30] which proposed several subsets of myalgic encephalomyelitis such as neurological, immune, metabolism/cardiorespiratory.

5. Conclusions

The association between occurrence of ME/CFS clinical symptoms, HHV-6, HHV-7 and B19 infection/coinfection reactivation and increased expression levels of TNF-α and IL-6 allows suggesting that these immunomodulating pathogens are involved in ME/CFS etiopathogenesis. Their role as trigger factors could not be excluded. The correlation of distinctive active viral infection with various types of clinical symptoms shows necessity of simultaneous study of these viral infections for identification of possible subsets of ME/CFS.

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References

[1] K. Fukuda, S. E. Straus, I. Hickie, M. C. Sharpe, J. G. Dobbins, and A. Komaroff, "The chronic fatigue syndrome: a comprehensive approach to its definition and study," *Annals of Internal Medicine*, vol. 121, no. 12, pp. 953–959, 1994.

[2] D. V. Ablashi, H. B. Eastman, C. B. Owen et al., "Frequent HHV-6 reactivation in multiple sclerosis (MS) and chronic

fatigue syndrome (CFS) patients," *Journal of Clinical Virology*, vol. 16, no. 3, pp. 179–191, 2000.

[3] S. Chapenko, A. Krumina, S. Kozireva et al., "Activation of human herpesviruses 6 and 7 in patients with chronic fatigue syndrome," *Journal of Clinical Virology*, vol. 37, supplement 1, pp. S47–S51, 2006.

[4] A. M. Lerner, S. H. Beqaj, R. G. Deeter, and J. T. Fitzgerald, "IgM serum antibodies to human cytomegalovirus nonstructural gene products p52 and CM2 (UL44 and UL57) are uniquely present in a subset of patients with chronic fatigue syndrome," *In Vivo*, vol. 16, no. 3, pp. 153–159, 2002.

[5] A. M. Lerner, S. H. Beqaj, R. G. Deeter, and J. T. Fitzgerald, "IgM serum antibodies to Epstein-Barr virus are uniquely present in a subset of patients with the chronic fatigue syndrome," *In Vivo*, vol. 18, no. 2, pp. 101–106, 2004.

[6] A. M. Lerner, S. H. Beqaj, R. G. Deeter, and J. T. Fitzgerald, "Valacyclovir treatment in Epstein-Barr virus subset chronic fatigue syndrome: thirty-six months follow-up," *In Vivo*, vol. 21, no. 5, pp. 707–713, 2007.

[7] J. R. Kerr, J. Bracewell, I. Laing et al., "Chronic fatigue syndrome and arthralgia following parvovirus B19 infection," *Journal of Rheumatology*, vol. 29, no. 3, pp. 595–602, 2002.

[8] M. Frémont, K. Metzger, H. Rady, J. Hulstaert, and K. De Meirleir, "Detection of herpesviruses and parvovirus B19 in gastric and intestinal mucosa of chronic fatigue syndrome patients," *In Vivo*, vol. 23, no. 2, pp. 209–213, 2009.

[9] M. Seishima, Y. Mizutani, Y. Shibuya, and C. Arakawa, "Chronic fatigue syndrome after human parvovirus B19 infection without persistent viremia," *Dermatology*, vol. 216, no. 4, pp. 341–346, 2008.

[10] J. K. S. Chia and A. Y. Chia, "Chronic fatigue syndrome is associated with chronic enterovirus infection of the stomach," *Journal of Clinical Pathology*, vol. 61, no. 1, pp. 43–48, 2008.

[11] A. S. Bansal, A. S. Bradley, K. N. Bishop, S. Kiani-Alikhan, and B. Ford, "Chronic fatigue syndrome, the immune system and viral infection," *Brain, Behavior, and Immunity*, vol. 26, no. 1, pp. 24–31, 2012.

[12] W. C. Reeves, F. R. Stamey, J. B. Black, A. C. Mawle, J. A. Stewart, and P. E. Pellett, "Human herpesviruses 6 and 7 in chronic fatigue syndrome: a case-control study," *Clinical Infectious Diseases*, vol. 31, no. 1, pp. 48–52, 2000.

[13] N. E. Soto and S. E. Straus, "Chronic fatigue syndrome and herpesviruses: the fading evidence," *Herpes*, vol. 7, no. 2, pp. 46–50, 2000.

[14] A. L. Komaroff, "Is human herpesvirus-6 a trigger for chronic fatigue syndrome?" *Journal of Clinical Virology*, vol. 37, supplement 1, pp. S39–S46, 2006.

[15] B. Cameron, L. Flamand, H. Juwana et al., "Serological and virological investigation of the role of the herpesviruses EBV, CMV and HHV-6 in post-infective fatigue syndrome," *Journal of Medical Virology*, vol. 82, no. 10, pp. 1684–1688, 2010.

[16] T. Saiki, T. Kawai, K. Morita et al., "Identification of marker genes for differential diagnosis of chronic fatigue syndrome," *Molecular Medicine*, vol. 14, no. 9-10, pp. 599–607, 2008.

[17] M. Bhattacharjee, C. H. Botting, and M. J. Sillanpää, "Bayesian biomarker identification based on marker-expression proteomics data," *Genomics*, vol. 92, no. 6, pp. 384–392, 2008.

[18] J. R. Kerr, B. Burke, R. Petty et al., "Seven genomic subtypes of chronic fatigue syndrome/myalgic encephalomyelitis: a detailed analysis of gene networks and clinical phenotypes," *Journal of Clinical Pathology*, vol. 61, no. 6, pp. 730–739, 2008.

[19] D. V. Ablashi, N. Balachandran, S. F. Josephs et al., "Genomic polymorphism, growth properties, and immunologic variations in human herpesvirus-6 isolates," *Virology*, vol. 184, no. 2, pp. 545–552, 1991.

[20] F. Santoro, P. E. Kennedy, G. Locatelli, M. S. Malnati, E. A. Berger, and P. Lusso, "CD46 is a cellular receptor for human herpesvirus 6," *Cell*, vol. 99, no. 7, pp. 817–827, 1999.

[21] Y. E. Cossart, A. M. Field, B. Cant, and D. Widdows, "Parvovirus like particles in human sera," *The Lancet*, vol. 1, no. 7898, pp. 72–73, 1975.

[22] K. E. Brown, S. M. Anderson, and N. S. Young, "Erythrocyte P antigen: cellular receptor for B19 parvovirus," *Science*, vol. 262, no. 5130, pp. 114–117, 1993.

[23] M. Söderlund-Venermo, K. Hokynar, J. Nieminen, H. Rautakorpi, and K. Hedman, "Persistence of human parvovirus B19 in human tissues," *Pathologie Biologie*, vol. 50, no. 5, pp. 307–316, 2002.

[24] L. Liefeldt, M. Buhl, B. Schweickert et al., "Eradication of parvovirus B19 infection after renal transplantation requires reduction of immunosuppression and high-dose immunoglobulin therapy," *Nephrology Dialysis Transplantation*, vol. 17, no. 10, pp. 1840–1842, 2002.

[25] L. B. Krupp, N. G. LaRocca, J. Muir-Nash, and A. D. Steinberg, "The fatigue severity scale. Application to patients with multiple sclerosis and systemic lupus erythematosus," *Archives of Neurology*, vol. 46, no. 10, pp. 1121–1123, 1989.

[26] P. Secchiero, D. R. Carrigan, Y. Asano et al., "Detection of human herpesvirus 6 in plasma of children with primary infection and immunosuppressed patients by polymerase chain reaction," *Journal of Infectious Diseases*, vol. 171, no. 2, pp. 273–280, 1995.

[27] Z. N. Berneman, D. V. Ablashi, G. Li et al., "Human herpesvirus 7 is a T-lymphotropic virus and is related to, but significantly different from, human herpesvirus 6 and human cytomegalovirus," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 89, no. 21, pp. 10552–10556, 1992.

[28] R. Cavallo, C. Merlino, D. Re et al., "B19 virus infection in renal transplant recipients," *Journal of Clinical Virology*, vol. 26, no. 3, pp. 361–368, 2003.

[29] F. Albright, K. Light, A. Light, L. Bateman, and L. A. Cannon-Albright, "Evidence for a heritable predisposition to chronic fatigue syndrome," *BMC Neurology*, vol. 11, article 62, 2011.

[30] B. M. Carruthers, M. I. Van de Sande, K. L. De Meirleir et al., "Myalgic encephalomyelitis: international consensus criteria," *Journal of Internal Medicine*, vol. 270, no. 4, pp. 327–338, 2011.

[31] M. Patnaik, A. L. Komaroff, E. Conley, E. A. Ojo-Amaize, and J. B. Peter, "Prevalence of IgM antibodies to human herpesvirus 6 early antigen (p41/38) in patients with chronic fatigue syndrome," *Journal of Infectious Diseases*, vol. 172, no. 5, pp. 1364–1367, 1995.

[32] D. M. Koelle, S. Barcy, M. L. Huang et al., "Markers of viral infection in monozygotic twins discordant for chronic fatigue syndrome," *Clinical Infectious Diseases*, vol. 35, no. 5, pp. 518–525, 2002.

[33] S. Kozireva, G. Nemceva, I. Danilane, O. Pavlova, J. Blomberg, and M. Murovska, "Prevalence of blood-borne viral infections (cytomegalovirus, human herpesvirus-6, human herpesvirus-7, human herpesvirus-8, human T-cell lymphotropic virus-I/II, human retrovirus-5) among blood donors in Latvia," *Annals of Hematology*, vol. 80, no. 11, pp. 669–673, 2001.

[34] M. Ihira, T. Yoshikawa, K. Suzuki et al., "Monitoring of active HHV-6 infection in bone marrow transplant recipients by real time PCR; comparison to detection of viral DNA in plasma by

qualitative PCR," *Microbiology and Immunology*, vol. 46, no. 10, pp. 701–705, 2002.

[35] P. Lusso, "HHV-6 and the immune system: mechanisms of immunomodulation and viral escape," *Journal of Clinical Virology*, vol. 37, supplement 1, pp. S4–S10, 2006.

[36] J. R. Kerr and D. A. J. Tyrrell, "Cytokines in parvovirus B19 infection as an aid to understanding chronic fatigue syndrome," *Current Pain and Headache Reports*, vol. 7, no. 5, pp. 333–341, 2003.

[37] M. A. Fletcher, X. R. Zeng, Z. Barnes, S. Levis, and N. G. Klimas, "Plasma cytokines in women with chronic fatigue syndrome," *Journal of Translational Medicine*, vol. 7, article 96, 2009.

[38] U. Vollmer-Conna, B. Cameron, D. Hadzi-Pavlovic et al., "Postinfective fatigue syndrome is not associated with altered cytokine production," *Clinical Infectious Diseases*, vol. 45, no. 6, pp. 732–735, 2007.

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Research Article

Inhibition of Indoleamine-2,3-dioxygenase (IDO) in Glioblastoma Cells by Oncolytic Herpes Simplex Virus

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Successful oncolytic virus treatment of malignant glioblastoma multiforme depends on widespread tumor-specific lytic virus replication and escape from mitigating innate immune responses to infection. Here we characterize a new HSV vector, JD0G, that is deleted for ICP0 and the joint sequences separating the unique long and short elements of the viral genome. We observed that JD0G replication was enhanced in certain glioblastoma cell lines compared to HEL cells, suggesting that a vector backbone deleted for ICP0 may be useful for treatment of glioblastoma. The innate immune response to virus infection can potentially impede oncolytic vector replication in human tumors. Indoleamine-2,3-dioxygenase (IDO) is expressed in response to interferon γ (IFNγ) and has been linked to both antiviral functions and to the immune escape of tumor cells. We observed that IFNγ treatment of human glioblastoma cells induced the expression of IDO and that this expression was quelled by infection with both wild-type and JD0G viruses. The role of IDO in inhibiting virus replication and the connection of this protein to the escape of tumor cells from immune surveillance suggest that IDO downregulation by HSV infection may enhance the oncolytic activity of vectors such as JD0G.

1. Introduction

Glioblastoma multiforme (GBM) is the most common type of primary brain tumor with an incidence rate of approximately 3 cases per 100,000 people per year in the United States (2004-2005, http://www.cbtrus.org/). The current treatment modality for GBM typically involves surgery to remove the tumor, followed by radiotherapy and adjuvant chemotherapy with temozolomide [1]. The infiltrative nature of the tumor makes complete surgical removal difficult, and tumor recurrence is common at the tumor margin. The median survival for patients with GBM is generally less than two years despite treatment. Clearly, new and effective therapies are needed.

Oncolytic vectors (OVs), viruses that have been designed to undergo lytic replication specifically in tumor cells, provide a novel therapeutic approach to treatment of GBM. Herpes simplex virus-based oncolytic vectors (oHSV) have been used in early-phase clinical trials for a variety of tumors including recurrent GBM. Recent trials using the genetically engineered HSV strains G207, HSV1716, OncoVEX$^{GM\text{-}CSF}$ and NV1020 have been encouraging, demonstrating that these viruses are relatively nontoxic for normal tissue and showing instances of promising patient responses [2–6]. However, despite their antitumor potential, the overall effectiveness of these vectors has been limited. This observation warrants the exploration of other mutant vector backbones that may improve tumor cell-dependent vector replication.

The engineering of an oHSV vector relies upon the deletion of viral genes that are needed to efficiently complete the virus life cycle in normal cells, but that are complemented by genetic alterations that commonly occur in tumor cells.

The ICP0 protein is an attractive target for the design of an oncolytic vector. ICP0 is a nonessential, immediate early gene product that performs a wide range of functions important for virus replication such as counteracting the IFN response [7–9], maintaining the viral genome in a transcriptionally active state [10–12], and targeting specific cellular proteins for degradation by the proteasome, including proteins important in modulating DNA repair pathways, apoptosis and cell-cycle progression [13–19]. Importantly, mutants lacking ICP0 protein are impaired for growth at low multiplicity of infection (MOI) in most cell lines [20], while vigorous replication has been observed in tumor cell lines such as U2OS osteosarcoma cells [21, 22]. It has recently been demonstrated that other tumor cell lines enhance the replication of ICP0-deficient vectors, and an HSV-1 mutant lacking ICP0 protein expression was shown to increase survival in a mouse breast adenocarcinoma model [23, 24]. The tumor cell-specific replication of ICP0-deficient HSV suggests that this mutant backbone may represent a new class of oHSV vector useful for treatment of GBM.

The innate immune response to virus infection can potentially inhibit virus replication and spread. For example, natural killer (NK) cells recruited to the site of HSV infection release interferon γ (IFNγ) a cytokine that triggers multiple downstream pathways that can inhibit virus replication [25]. The production of IFNγ has been shown to inhibit replication of certain oHSV *in vitro* and virus gene expression *in vivo* [25, 26]. Local production of IFNγ induces the synthesis of indoleamine-2,3-dioxygenase (IDO), an enzyme that catalyzes the degradation of the essential amino-acid tryptophan producing kynurenine and multiple downstream metabolites. The consequent depletion of tryptophan from the cell and its surroundings impedes virus replication [27]. Furthermore, kynurenine along with other catabolites has been linked to the escape of tumor cells from immune surveillance [28–30]. Thus, IDO may present obstacles to both OV replication and immune clearance of tumor cells.

In this report, we characterized an ICP0-deficient oHSV vector, JD0G [31], for its ability to replicate in glioblastoma cells in the absence and presence of IFNγ. We show that JD0G virus replication was enhanced in glioblastoma cell lines compared to HEL cells, suggesting that the former complement the loss of ICP0 protein. Both wild-type KOS virus and JD0G were moderately sensitive to IFNγ at low MOI in glioblastoma cells, an effect that was dramatically reduced at higher MOI. Moreover, while glioblastoma cell lines produced IDO protein when stimulated with IFNγ, IDO induction had a minimal effect on virus replication. This observation may be related to our finding that IFNγ-induced IDO levels were substantially reduced in glioma cells infected with KOS or JD0G

2. Results

2.1. JD0G Structure. To create JD0G, the internal repeat sequences "joint" were deleted from the strain KOS HSV-1 genome, a modification that removes one copy each of the gamma 34.5 gene and the immediate early genes encoding ICP0 and ICP4. The removal of the joint enhances vector stability by eliminating internal genome rearrangements that occur during U_L/U_S isomerization [38]. The joint deletion removes a region spanning positions from 116,982 to 132,605 of the HSV genome (nucleotide positions based on NC_001806). The remaining copy of ICP0 was then deleted, and an eGFP expression construct driven by the human cytomegalovirus (hCMV) major immediate early promoter was inserted in its place. The structure of the mutant virus was confirmed by PCR, sequencing, and Southern blot analysis. Figure 1 shows the structure of the JD0G genome compared to that of wild-type HSV-1 KOS.

2.2. Glioblastoma Cell Lines Support Replication of the JD0G Virus. Successful oncolytic virus treatment of GBM requires efficient, tumor-specific replication, and lysis of the tumor cell. We compared JD0G replication with that of wild-type KOS virus and another oncolytic vector, MGH2, a derivative of the well-studied vector G207 [32]. MGH2 lacks expression of γ34.5 and produces a nonfunctional ICP6 protein fused with GFP from the U_L39 locus. In addition, MGH2 carries two genes that encode prodrug activating enzymes, *CYP2B1* (cytochrome p450), and *shiCE* (secreted human intestinal carboxylesterase). ICP0-deficient viruses are impaired for replication at low MOI in cells such as human embryonic lung fibroblasts (HEL cells), while certain tumor cell lines have been shown to allow efficient replication [22]. To assess the ability of human glioblastoma cell lines to support virus replication, HEL, SNB19, and U251 glioblastoma cells were infected at an MOI of 0.1 (based on U2OS titers), supernatants were collected at 24 hours postinfection (hpi) and virus yields were measured by titration on U2OS cells. Infections with KOS consistently generated slightly higher virus yields on HEL cells than on the two glioma cell lines (Figure 2(a)). In contrast, infection of the glioma cells with JD0G virus produced titers that were on average 10-fold higher than those obtained from HEL cells (Figure 2(a)). A similar comparison of MGH2 titers across the cell lines demonstrated that while MGH2 was able to replicate in HEL cells, its replication was substantially more impaired than that of JD0G on the two glioblastoma cell lines (Figure 2(a)). These results suggest that both glioblastoma cell lines provided an enhanced environment for JD0G growth, but not for MGH2, and that these tumor cells partially complement the ICP0 deficit.

We then examined the ability of the JD0G and MGH2 oncolytic viruses to cause tumor-specific cell death. HEL, SNB19, and U251 cells were infected at an MOI of 0.1, and the surviving cells were counted at 48 hpi (Figure 2(b)). Compared to mock-infected HEL cells, JD0G infection reduced the number of HEL cells by less than 2-fold. In contrast, the number of glioma cells that survived infection with JD0G was reduced by 10-fold or greater. MGH2 infection did not exhibit similar tumor-specific cell-killing activity, causing more cell death in the HEL cell line than in either glioma cell line. Thus, the JD0G oncolytic vector both exhibited a better tumor-selective replication profile than MGH2 and demonstrated clear glioma-specific cell death.

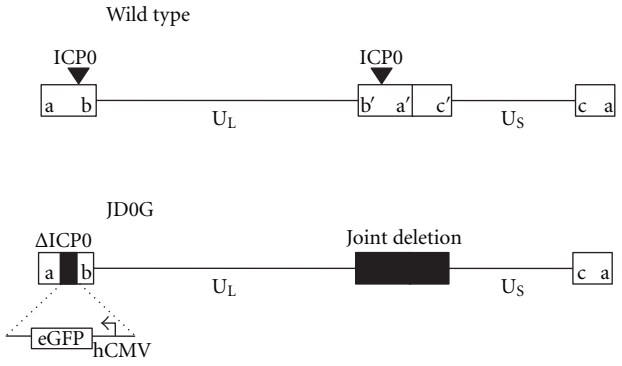

Figure 1: Structure of the JD0G virus compared to the wild-type HSV-1 virus. Schematics depicting the genome structure of wild-type HSV-1 (top) and the JD0G virus (bottom). The unique long (U_L) and unique short (U_S) portions of the viral genome are indicated with solid lines, and the repeat regions of the genome (ab/b′a′, a′c′/ca) are shown as open boxes. The locations of the two ICP0 loci are designated with inverted triangles. Black boxes represent deleted regions of the JD0G genome. The joint deletion removes a region spanning positions from 116,982 to 132,605 of the HSV genome (nucleotide positions based on NC_001806). The terminal ICP0-deletion (ΔICP0) replaces the ICP0 coding sequence with an hCMV:eGFP expression cassette.

2.3. IFNγ Treatment Inhibits Virus Replication in an MOI-Dependent Manner. IFNγ presents a potential impediment to virus replication in tumor cells *in vivo*. To evaluate the sensitivity of KOS and JD0G to type II IFN, SNB19 and U251 cells were treated with IFNγ for 24 h and infected with either KOS or JD0G at an MOI of 0.1 or 10. Virus yields at 24 hpi were assessed by titration on U2OS cells. The results (Figures 3(a) and 3(b)) showed that (i) growth of KOS and JD0G was inhibited by IFNγ at low MOI on both cell lines; (ii) JD0G was somewhat more sensitive than KOS; (iii) both viruses at low MOI were hypersensitive to IFNγ on U251 cells; and (iv) high-MOI infection minimized IFNγ sensitivity. These results suggested that ICP0 deficiency has a moderate (≤5-fold) effect on IFNγ sensitivity at low MOI, a condition that likely models *in vivo* infection more realistically than high-MOI infection of cultured cells. In addition, the data indicated that both the antiviral response to IFNγ and the contribution of ICP0 to viral escape from this response at high MOI vary between glioblastoma lines.

2.4. IFNγ-Induced IDO Protein Has a Minimal Effect on Virus Replication. Local production of IFNγ induces the synthesis of indoleamine-2,3-dioxygenase (IDO), an enzyme that catalyzes degradation of the essential amino acid tryptophan producing kynurenine. IDO production in response to IFNγ has been linked to the inhibition of HSV replication in certain cell lines *in vitro* [27]. In order to determine if the glioblastoma cells used in this study produce active IDO protein either constitutively or in response to IFNγ, IDO-enzymatic activity was assessed by measuring the level of kynurenine in the supernatants of IFNγ-treated cells. Kynurenine levels increased in a dose-dependent manner in response to increasing levels of IFNγ (Figure 4(a)). These

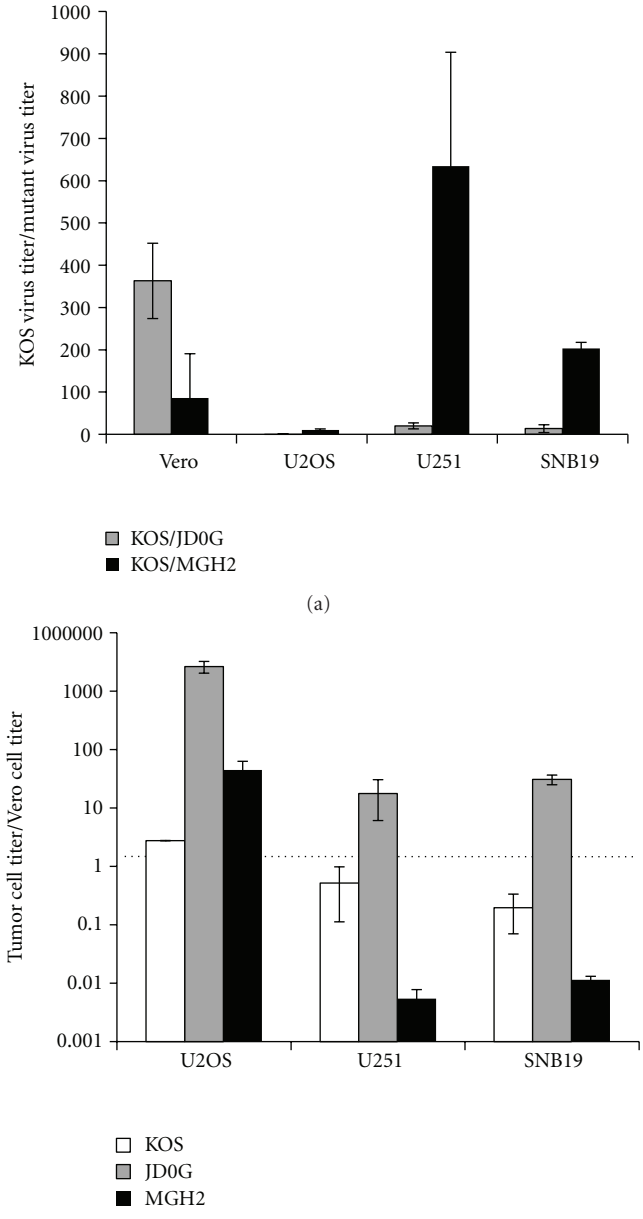

Figure 2: JD0G infection of glioblastoma cells results in efficient virus replication and tumor-selective cell death. (a) The replication of JD0G was evaluated in comparison to KOS and MGH2. HEL, U251, and SNB19 cell lines were infected in parallel with KOS, MGH2, or JD0G at an MOI of 0.1 based on U2OS titers. Supernatants were collected at 24 hpi, and the amount of virus produced was assessed by titration on U2OS cells. The data represent the average of three independent infections, and error bars represent the standard deviation. For all three viruses the differences in virus yield observed between HEL cells and either glioma cell line are statistically significant as determined by the student *t*-test (*P* values less than 0.03). (b) Tumor-specific cell death was assessed by counting the number of surviving cells following infection with either JD0G or MGH2 virus. HEL, U251 or SNB19 cells were infected at an MOI of 0.1, and Trypan Blue staining was performed to count the number of viable cells 48 h postinfection. The data represent the average of three independent infections, and error bars represent the standard deviation. All differences are statistically significant (*P* values less than 0.01).

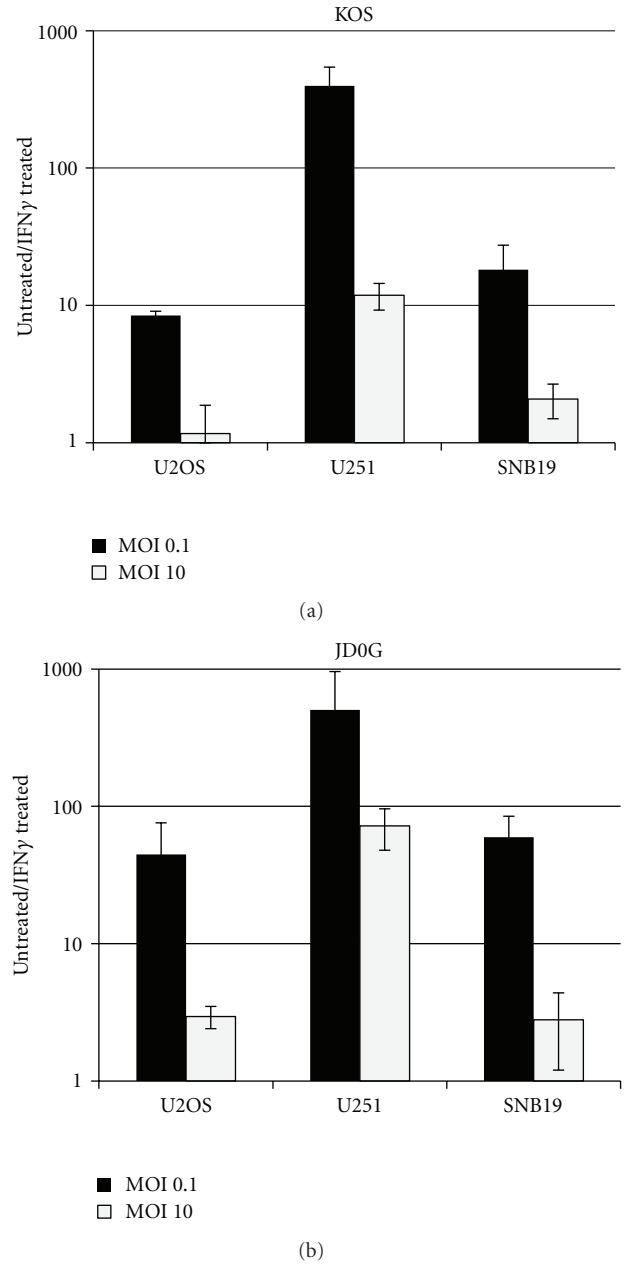

Figure 3: IFNγ treatment of glioblastoma cells results in a decrease in virus yield in an MOI-dependent manner. U251 and SNB19 cells were pretreated or mock treated with IFNγ (500 U/mL) 24 h prior to infection with either KOS or JD0G at an MOI of 0.1 (a) or 10 (b), and the supernatants were titered at 24 hpi. The data present the average of two independent infections, and the error bars represent the standard deviation; these data are representative of replicate experiments.

data illustrated that active IDO protein was produced in response to IFNγ treatment of glioblastoma cells.

We used the small molecule inhibitor of IDO activity, 1-methyl-tryptophan (1-MT), to assess the role of IDO in the observed reduction in virus yields from glioblastoma cells treated with IFNγ prior to low-MOI infection (Figure 4(b)). In U251 cells, IFNγ decreased both KOS and JD0G yields over 100-fold, and the addition of 1-MT consistently reduced this loss to approximately 15-fold; 1-MT alone had no effect on virus growth (data not shown). In contrast, the presence of 1-MT did not significantly alter the reduced yields of KOS and JD0G on IFNγ-treated SNB19 cells. These data suggested that IDO contributes to the IFNγ hypersensitivity of the two viruses on U251 cells, but not to their lower sensitivity on SNB19 cells. In both instances, however, the main effect of IFNγ appeared to be mediated via a different pathway.

2.5. Virus Infection Reduces the Amount of IDO mRNA and Protein in IFNγ-Treated Cells. Although active IDO protein was produced in response to IFNγ in both U251 and SNB19 cells, the 1-MT experiments described above indicated that IDO activity did not significantly influence virus replication in at least one of these lines, SNB19. This may suggest that HSV-1 can counteract the IDO pathway to allow virus replication. The level of IDO protein present in U251 and SNB19 cells was measured following IFNγ treatment and virus infection (Figure 5(a)). Untreated cells and cells infected with virus alone did not show detectable levels of IDO protein at 12 and 24 hpi (Figure 5(a)). Cells treated with IFNγ produced IDO protein in both cell lines. However, cells treated with IFNγ and then infected with either KOS or JD0G virus showed a substantial reduction in the amount of IDO protein (Figures 5(a) and 5(b)). At 24 hpi, less than 20% of the IDO protein remained in SNB19 cells and less than 30% in U251 cells (Figure 5(b)). These data, representative of 3 independent experiments, suggested that infection of cells with HSV-1 can lead to downregulation of IDO protein.

Downregulation of IDO in response to virus infection can occur either at the protein or mRNA level. We therefore measured IDO mRNA expression by quantitative RT-PCR at 2 and 8 hours following virus infection of IFNγ-treated cells. The results (Figure 5(c)) demonstrated that IDO mRNA levels were reduced as early as 2 hours postinfection with either KOS or JD0G viruses when compared to mock-treated controls. Together these data suggest that infection of glioblastoma cells with HSV results in reduced expression of both IDO mRNA and protein, thereby minimizing the inhibitory effects of this protein on virus replication.

3. Discussion

The development of attenuated viruses that are adapted for preferential replication in solid tumors is an attractive approach to treatment of malignancies where more standard therapies are either ineffective or difficult to apply. HSV-1-oncolytic vectors used in early-phase clinical trials for the treatment of GBM have shown some success without serious side effects [33, 34]. Tumor specificity can be achieved by deleting viral genes that permit mutant virus replication in tumor cells while profoundly impairing virus replication in normal host cells [1]. The vector prototype is G207, that is, deleted for γ34.5 and produces a nonfunctional ICP6-LacZ fusion protein [35]. ICP6 encodes the large subunit of the viral ribonucleotide reductase, a protein that permits virus growth in nondividing cells by maintaining the nucleotide

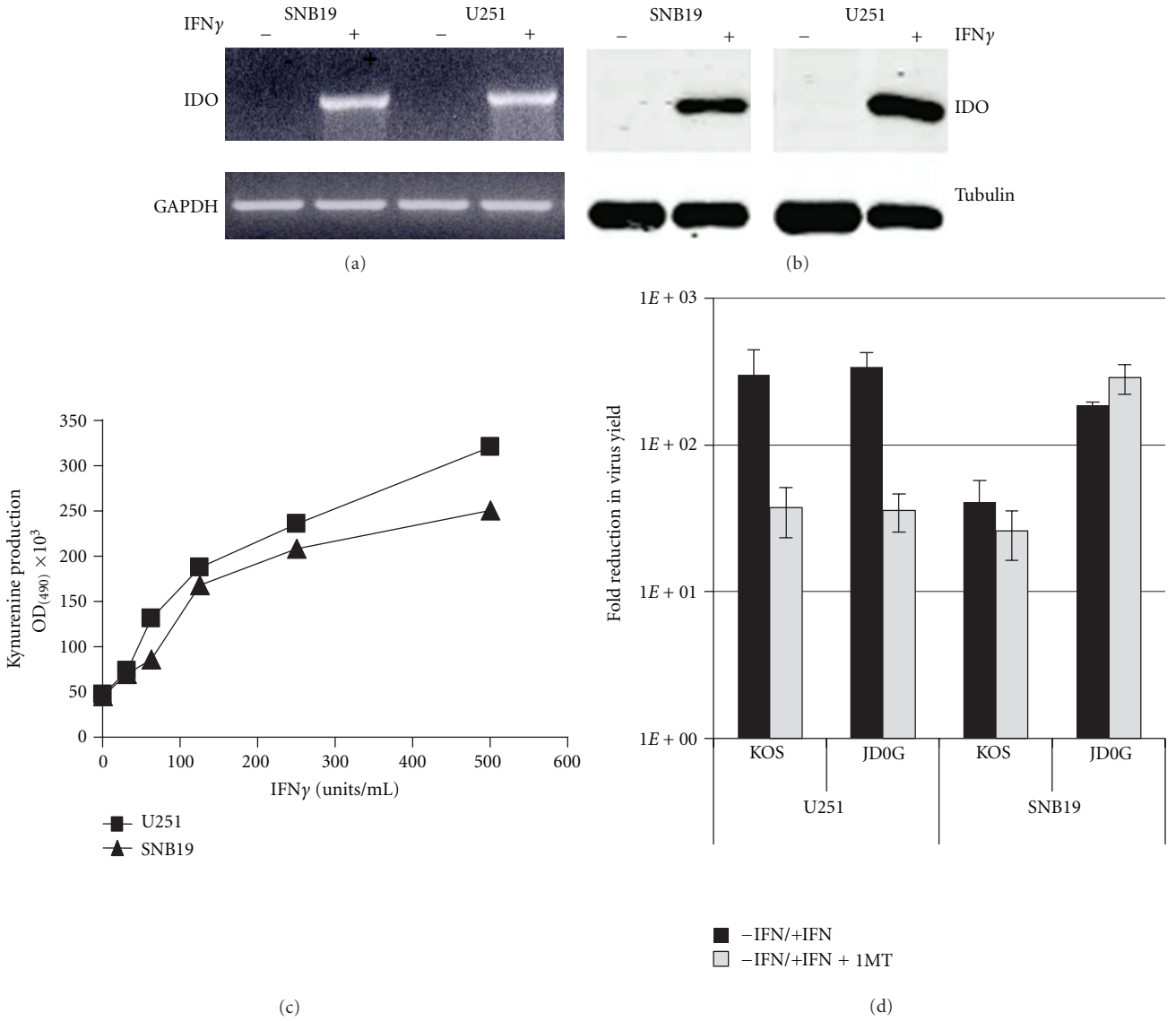

Figure 4: IFNγ-induced IDO protein has a minimal effect on virus replication. (a) SNB19 or U251 cells were pretreated with varying concentrations of IFNγ (0–500 U/mL) in media supplemented with 100 μg/mL of L tryptophan. After 3 days of incubation, IDO activity was measured by determining kynurenine content in the cell supernatants. (b) U251 or SNB19 cell lines were pretreated or mock treated with IFNγ (500 U/mL) with or without 1-MT (300 μM) 24 h prior to infection with JD0G or KOS virus, and the supernatants were titered at 24 hpi. The data represent the average of two independent infections, and the error bars represent the standard deviation; these data are representative of replicate experiments.

pool, while γ34.5 counteracts the virus-induced activation of the PKR pathway. The more advanced oncolytic vector examined in this study, MGH2, was derived from G207 by replacing *lacZ* with eGFP at the ICP6 locus and inserting two antitumor genes, *CYP2B1* (encoding cytochrome p450) and *shiCE* (encoding secreted human intestinal carboxylesterase) [32]. These enzymes activate the anticancer drugs cyclophosphamide and irinotecan, respectively, both of which are potent tumor toxic products. In one study, MGH2 showed oncolytic activity *in vivo* only with the addition of cyclophosphamide and irinotecan [32], indicating that the MGH2 vector backbone alone does not function as an effective OV. Some evidence suggests that vector replication in certain tumor cells may require γ34.5 activity and that oHSV is susceptible to innate immune responses, potentially limiting the effectiveness of this and other oHSV vectors [36]. We therefore sought to examine other mutant backbones that may overcome these limitations.

In this study we characterize an oHSV vector (JD0G) that is deleted for ICP0 and the joint elements of the viral genome. HSV-1 mutants defective for the production of ICP0 protein provide an attractive alternative to the MGH2 vector backbone. ICP0-deficient vectors are impaired for growth at low MOI in most cell lines, while replication preferentially occurs in certain tumor cell lines [22, 23, 37]. Moreover, Hummel and colleagues have reported that

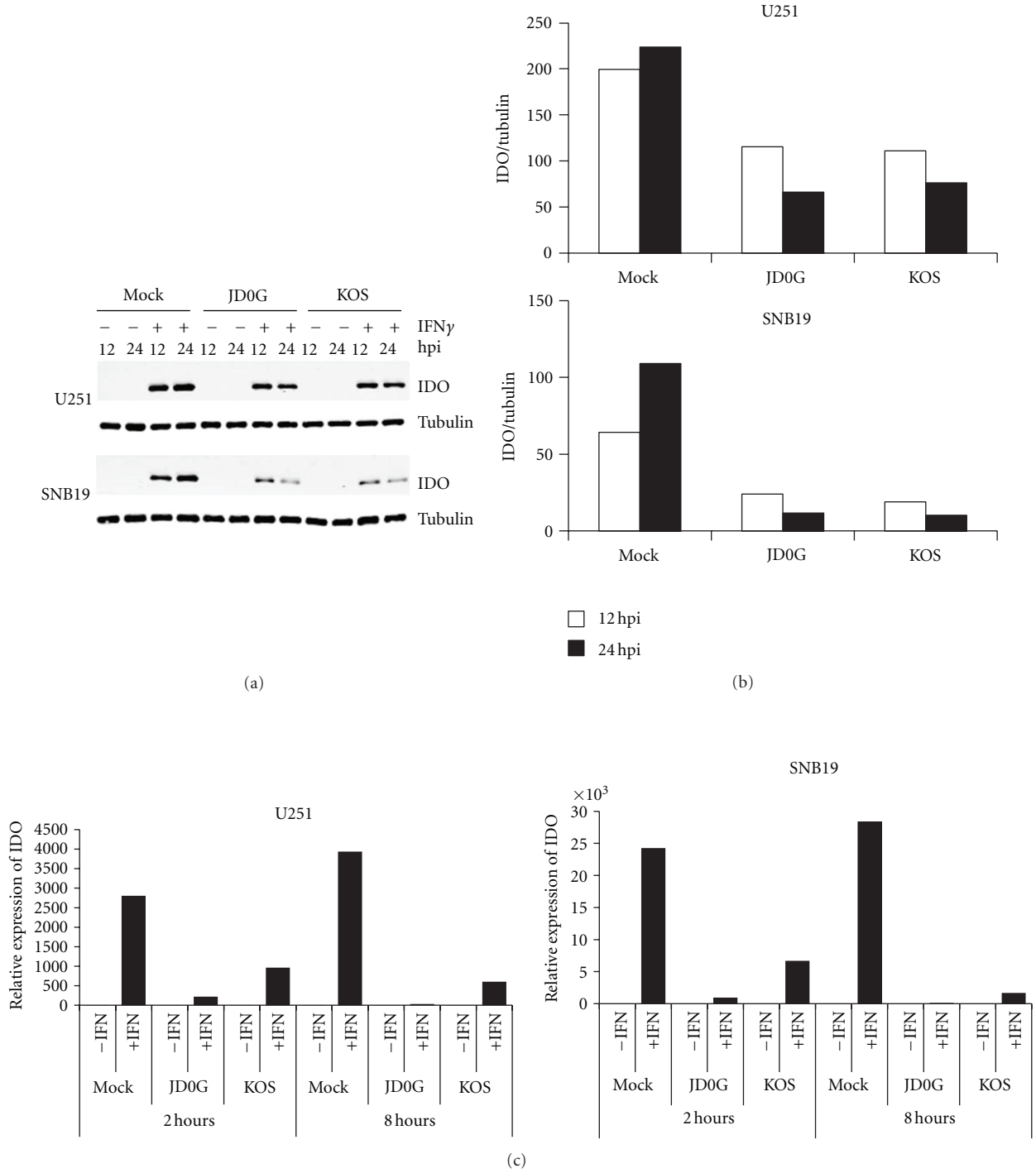

Figure 5: Reduction in IFNγ-induced IDO expression following virus infection of glioblastoma cells. (a) The effect of virus infection on IFNγ-induced IDO protein levels in SNB19 cells (top) and U251 cells (bottom) was visualized by Western blot. Cells were treated with IFNγ or mock treated for 24 h prior to infection with either JD0G or KOS virus. At 12 and 24 hpi, cell lysates were analyzed by Western blot with antibodies against IDO protein (upper panels) or α-tubulin (lower panels). (b) IDO protein levels were quantified using the Odyssey Infrared Imaging System, and the relative amount of IDO protein (IDO/α-tubulin) in each sample is shown. Data are representative of three experiments using different time points. (c) The effect of virus infection on IDO mRNA levels in SNB19 and U251 cells was assessed by quantitative RT-PCR. Cells were treated with IFNγ or mock treated for 24 h prior to infection with either JD0G or KOS virus, and total cellular RNA was collected at 2 and 8 hpi. IDO mRNA levels for each sample were normalized to GAPDH levels and the fold change in IDO expression for each sample is shown relative to the untreated, mock-infected control.

an HSV-1 double mutant lacking VP16 and ICP0 protein expression replicates efficiently in a variety of tumor cells derived from prostate, lung, colon, and mammary carcinomas with evidence of oncolytic activity in animal models [24]. Using the JD0G backbone, we sought to determine whether the unique interaction of an ICP0-deleted vector with tumor cells could be exploited to develop novel oncolytic viruses suitable for treatment of brain tumors. Our findings showed that JD0G replication was significantly enhanced in the U251 and SNB19 glioblastoma cell lines compared to HEL cells, while MGH2 replication was highly impaired, showing 2-3 logs lower yields on the glioblastoma lines than JD0G. These findings point to the possibility that genetic changes related to glioblastoma development relieve the need for ICP0 expression and permit JD0G replication, while the mutations in MGH2 are detrimental to vector growth and not complemented in these lines. Furthermore, the difference in replication between these two vectors was most pronounced at low multiplicity (data not shown), a condition that may be more relevant to circumstances *in vivo* than high-MOI infections in cell culture. An oHSV vector that can replicate efficiently from a small number of initial infectious particles is most likely to be effective in tumors.

It is likely that the tumor-specific replication of the JD0G vector can be ascribed to the ICP0 deficiency and not to the removal of the joint components of the viral genome. The HSV genome consists of two unique elements (U_L and U_S) that are each flanked by repeated sequences (ab/b′a′; a′c′/ca) (Figure 1). This arrangement creates two terminal repeat regions that together contain one copy of the genes for ICP34.5, ICP4, ICP0, and the latency-associated transcript (LAT), and an internal repeat element (joint) that contains a second copy of each gene. Thus, the joint deletion generated in the construction of JD0G removes a single copy of each gene, reducing but not eliminating gene expression. This modification alone has a minimal effect on virus replication (unpublished observations, [38]). The benefit of this deletion is that it eliminates the possibility of genome isomerization events that typically occur between the repeated elements of the viral genome and generate four possible HSV isomers. Hence, the JD0G genome has enhanced stability over the wild-type KOS genome.

Following inoculation of oHSV *in vivo,* IFNγ can be produced by resident microglia, recruited macrophages, dendritic and NK cells at the growing tumor site [25, 39]. In response to IFNγ, dendritic cells and tumor cells may produce IDO, whose enzymatic activity leads to the degradation of tryptophan and the production of toxic catabolites such as kynurenine and quinolinate [40]. Additionally, tumor cells including glioblastoma cells have been reported to express IDO without IFNγ induction [41]. Tryptophan depletion can impede the production of viral proteins and, together with kynurenine, can induce effector T-cell anergy [30]. Therefore, IFNγ can negatively affect both oncolytic virus replication and tumor-specific immunity at several levels. Although IFNγ-mediated inhibition of oHSV replication may be overcome in part by the administration of immunosuppressive drugs such as cyclophosphamide, a drawback to this approach is that it will also inhibit the induction of tumor-specific immunity.

In view of these considerations, and to explore the tumor-related changes that may potentially influence the innate immune response to virus infection, KOS and JD0G viral replication in glioblastoma cells were tested for their sensitivity to IFNγ treatment. Our data demonstrate that both viruses were sensitive to IFNγ at low MOI, but the ability of IFNγ to control virus replication was largely lost at elevated MOI. However, we observed marked differences between the glioblastoma cell lines in both the IFNγ sensitivity of virus replication and the involvement of IDO activity in the inhibition of virus replication. SNB19 cells produced lower levels of IDO protein than U251 cells, and virus replication in SNB19 cells was less sensitive to IFNγ treatment. In the IFNγ-hypersensitive U251 cells, both KOS and JD0G were found to downregulate, but not eliminate, IDO mRNA and protein, and the IDO inhibitor 1-MT was able to improve the efficiency of virus replication. In contrast, both KOS and JD0G infection nearly eliminated IDO expression in IFNγ-treated SNB19 cells, and 1-MT did not improve virus replication. Together, these results indicate that IDO expression above a certain level can inhibit virus replication in glioblastoma cells, but also that the majority of IFNγ-induced inhibition of virus replication occurs via a different pathway. The limited role of IDO in controlling virus replication may be attributable to the virus-mediated downregulation of IDO observed on infection with both KOS and JD0G. This downregulation may not only allow for vector replication in the presence of IFNγ, but also minimize adverse effects on antitumor immunity. Thus, we propose that vector-induced IDO downregulation may be an important aspect of the therapeutic potential of oHSV vectors.

4. Methods

4.1. Cell Lines and Viruses. Human glioblastoma SNB19 and U251 [42], osteosarcoma U2OS (ATCC, Manassas, VA, USA), embryonic lung HEL (ATCCs), and monkey kidney Vero cells (ATCC) were cultured by standard methods. HEL cells were maintained in RPMI supplemented with 10% fetal bovine serum (FBS). All other cells were maintained in Dulbecco's Modified Eagle Medium (Atlanta Biologicals, Norcross, GA, USA) supplemented with 10% FBS at 37°C in 5% CO_2. The wild-type HSV-1 (KOS) virus stock was prepared on Vero cells.

The JD0G mutant HSV-1 virus was derived by deletion of ICP0 by mixed infection/transfection of the Joint Deleted vector with plasmid 28E3. In order to obtain 28E.3 we initially deleted the fragment StuI/HpaI from the plasmid 28.1 (which consists of the Dra I fragment of psg28 [43], and a Bgl II linker was inserted into this site. Plasmid 28E3 was created by cloning a Bgl II fragment containing a HCMV eGFP expression construct from pEGFPN-1 (Clontech, Palo Alto, CA, USA) into the Bgl II site of 28B.

JD0G virus stocks were prepared on U2OS cells. The MGH2 virus was kindly provided by Antonio Chiocca (Ohio State University). For comparison purposes, MOIs were based throughout on viral titers determined on U2OS cells.

4.2. Virus Growth. 1×10^5 cells were seeded in 24-well dishes prior to infection. Confluent monolayers of cells were infected at an MOI of 0.1 in serum-free DMEM (Atlanta Biologicals) for 2 h. Supernatants were collected at 24 hpi, and viral titers were determined by serial dilution on U2OS cells. Plaques were visualized by crystal violet staining.

4.3. IFNγ Treatment. 7×10^4 cells were seeded in 24-well dishes and pretreated or mock treated for 24 h with 500 U/mL of recombinant human IFNγ (Cell Sciences, Canton, MA, USA). The IDO inhibitor 1-methyl-L-tryptophan (Sigma, St. Louis, MO, USA) was used at a final concentration of 300 μM. Pretreated cells were infected at the appropriate MOI in serum-free DMEM for 2 h, virus was removed, and the cells were fed with fresh media plus IFNγ. Supernatants were collected at 24 hpi and viral titers were determined by serial dilution on U2OS cells. Plaques were visualized by crystal violet staining.

4.4. Quantitative RT-PCR. 3×10^5 cells were seeded in 6-well dishes and pretreated or mock treated for 20 h with 500 U/mL of IFNγ. Pretreated cells were infected at an MOI of 20 in serum-free DMEM with either KOS or JD0G for 2 h, virus was removed, and fresh media with IFNγ was added to the wells. Cells were harvested at the indicated times postinfection (either 2 or 8 h), and total RNA was extracted using the RNeasy kit (QIAGEN, Valencia, CA, USA). 2 μg of total RNA was used to synthesize the first cDNA strand using Superscript First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Quantitative PCR was performed as previously described [44]. Average C_T values for IDO were normalized with average C_T values for GAPDH, and data were plotted as the fold change relative to untreated, mock-infected controls using the $2^{-\Delta\Delta C_T}$ method [45]. The following primers and probes were obtained from PE Applied Biosystems (Foster City, CA, USA): IDO Fwd, GGTCATGGAGATGTCCGTAA; IDO Rev, ACCAATAGAGAGACCAGGAAGAA; IDO Probe, 6FAM-CTGTTCCTTACTGCCAACTCTCCAAGAAACTG-Tamra; GAPDH-FWD, CCCCACACACATGCACTTACC; GAPDH-REV, CCTACTCCCAGGGCTTTGATT; GAPDH-Claw-Probe, 6FAM-AAAGAGCTAGGAAGGACAGGC-AACTTGGC-TAMRA.

4.5. Kynurenine Assay. 3×10^4 cells were pretreated with varying concentrations of IFNγ (0–500 U/mL) in media supplemented with 100 μg/mL L-tryptophan. After 3 days of incubation, supernatants were harvested for determination of kynurenine content. Samples of 160 μL were incubated with 10 μL of 30% TCA for 30 min at 50°C and centrifuged at 3,000 rpm for 20 min at room temperature. 100 μL of each sample was reacted with an equal volume of Ehrlich reagent (Sigma) at room temperature for 10 min, and the OD_{490} measured using a microplate reader.

4.6. Western Blot. 3×10^5 cells were seeded in 6-well dishes and pretreated or mock treated for 20 h with 500 U/mL of IFNγ. Pretreated cells were infected at an MOI of 20 in serum-free DMEM with either KOS or JD0G for 2 h, virus was removed, and fresh media with IFNγ was added to the wells. Cells were harvested at the indicated times postinfection (either 12, or 24 h), resuspended in 200 μL lysis buffer (10 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA pH 8, 0.1% Triton-X, 1 mM DTT, 1 mM PMSF, 1 mM $NaVO_4$, and 0.2% Protease Inhibitor Cocktail [Sigma]), and incubated on ice for 30 min. 5 μg of total protein was resolved on an 8% SDS-PAGE gel and transferred to Protran 0.45 μm nitrocellulose membrane (Whatman, Piscataway, NJ, USA). The membrane was blocked for 1 h at room temperature in Tween-20-free Odyssey blocking buffer (LI-Cor Biosciences, Lincoln, NE, USA) and incubated overnight at 4°C with a 1 : 5,000 dilution of ALX-210-429 rabbit anti-IDO antibody (Alexis Biochemicals, Lausanne, Switzerland) and a 1 : 20,000 dilution of mouse anti-α-tubulin antibody (T6074, Sigma) in 1x PBS/0.05% Tween-20. The membrane was washed with 1x PBS/0.05% Tween-20, incubated for 1 h at room temperature with goat anti-mouse IRDye800CW-green and goat anti-rabbit IRDye680-red secondary antibodies (both from LI-Cor), and washed with 1x PBS/0.05% Tween-20. The membrane was scanned with Odyssey Infrared Imaging System (LI-Cor) and analyzed with Odyssey 3.0 software as specified by the manufacturer.

4.7. Cell Killing. 1×10^6 cells were seeded in 6-well dishes and infected 24 hours later at an MOI of 0.1 in serum-free DMEM (Atlanta Biologicals). Media was replaced with fresh serum-containing media after 1.5 h and 48 h postinfection viable cells were stained with Trypan Blue and counted.

Conflict of Interests

The authors declare no conflict of interests.

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References

[1] P. Grandi, P. Peruzzi, B. Reinhart, J. B. Cohen, E. A. Chiocca, and J. C. Glorioso, "Design and application of oncolytic HSV vectors for glioblastoma therapy," *Expert Review of Neurotherapeutics*, vol. 9, no. 4, pp. 505–517, 2009.

[2] J. C. Hu, M. J. Booth, G. Tripuraneni et al., "A novel HSV-1 virus, JS1/34.5-/47—purges contaminating breast cancer cells from bone marrow," *Clinical Cancer Research*, vol. 12, no. 22, pp. 6853–6862, 2006.

[3] J. M. Markert, M. D. Medlock, S. D. Rabkin et al., "Conditionally replicating herpes simplex virus mutant G207 for the treatment of malignant glioma: results of a phase I trial," *Gene Therapy*, vol. 7, no. 10, pp. 867–874, 2000.

[4] V. Papanastassiou, R. Rampling, M. Fraser et al., "The potential for efficacy of the modified (ICP 34.5-) herpes simplex virus HSV1716 following intratumoural injection into malignant glioma: a proof of principle study," *Gene Therapy*, vol. 9, no. 6, pp. 398–406, 2002.

[5] R. Rampling, G. Cruickshank, V. Papanastassiou et al., "Toxicity evaluation of replication-competent herpes simplex virus (ICP 34.5 null mutant 1716) in patients with recurrent malignant glioma," *Gene Therapy*, vol. 7, no. 10, pp. 859–866, 2000.

[6] N. N. Senzer, H. L. Kaufman, T. Amatruda et al., "Phase II clinical trial of a granulocyte-macrophage colony-stimulating factor-encoding, second-generation oncolytic herpesvirus in patients with unresectable metastatic melanoma," *Journal of Clinical Oncology*, vol. 27, no. 34, pp. 5763–5771, 2009.

[7] R. D. Everett and A. Orr, "Herpes simplex virus type 1 regulatory protein ICP0 aids infection in cells with a preinduced interferon response but does not impede interferon-induced gene induction," *Journal of Virology*, vol. 83, no. 10, pp. 4978–4983, 2009.

[8] W. P. Halford, C. Weisend, J. Grace et al., "ICP0 antagonizes Stat 1-dependent repression of herpes simplex virus: implications for the regulation of viral latency," *Virology Journal*, vol. 3, article 44, 2006.

[9] D. A. Leib, T. E. Harrison, K. M. Laslo, M. A. Machalek, N. J. Moorman, and H. W. Virgin, "Interferons regulate the phenotype of wild-type and mutant herpes simplex viruses in vivo," *Journal of Experimental Medicine*, vol. 189, no. 4, pp. 663–672, 1999.

[10] H. Gu and B. Roizman, "Herpes simplex virus-infected cell protein 0 blocks the silencing of viral DNA by dissociating histone deacetylases from the CoREST-REST complex," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 43, pp. 17134–17139, 2007.

[11] W. Cai and P. A. Schaffer, "Herpes simplex virus type 1 ICP0 regulates expression of immediate-early, early, and late genes in productively infected cells," *Journal of Virology*, vol. 66, no. 5, pp. 2904–2915, 1992.

[12] J. Chen and S. Silverstein, "Herpes simplex viruses with mutations in the gene encoding ICP0 are defective in gene expression," *Journal of Virology*, vol. 66, no. 5, pp. 2916–2927, 1992.

[13] C. Boutell, R. Everett, J. Hilliard, P. Schaffer, A. Orr, and D. Davido, "Herpes simplex virus type 1 ICP0 phosphorylation mutants impair the E3 ubiquitin ligase activity of ICP0 in a cell type-dependent manner," *Journal of Virology*, vol. 82, no. 21, pp. 10647–10656, 2008.

[14] C. Boutell, A. Orr, and R. D. Everett, "PML residue lysine 160 is required for the degradation of PML induced by herpes simplex virus type 1 regulatory protein ICP0," *Journal of Virology*, vol. 77, no. 16, pp. 8686–8694, 2003.

[15] C. Boutell, S. Sadis, and R. D. Everett, "Herpes simplex virus type 1 immediate-early protein ICP0 and its isolated RING finger domain act as ubiquitin E3 ligases in vitro," *Journal of Virology*, vol. 76, no. 2, pp. 841–850, 2002.

[16] M. K. Chelbi-Alix and H. De Thé, "Herpes virus induced proteasome-dependent degradation of the nuclear bodies-associated PML and Sp100 proteins," *Oncogene*, vol. 18, no. 4, pp. 935–941, 1999.

[17] R. D. Everett, "Herpes simplex virus type 1 regulatory protein ICP0 does not protect cyclins D1 and D3 from degradation during infection," *Journal of Virology*, vol. 78, no. 18, pp. 9599–9604, 2004.

[18] R. Lin, R. S. Noyce, S. E. Collins, R. D. Everett, and K. L. Mossman, "The herpes simplex virus ICP0 RING finger domain inhibits IRF3- and IRF7-mediated activation of interferon-stimulated genes," *Journal of Virology*, vol. 78, no. 4, pp. 1675–1684, 2004.

[19] P. Lomonte, K. F. Sullivan, and R. D. Everett, "Degradation of nucleosome-associated centromeric histone H3-like protein CENP-A induced by herpes simplex virus type 1 protein ICP0," *Journal of Biological Chemistry*, vol. 276, no. 8, pp. 5829–5835, 2001.

[20] N. D. Stow and E. C. Stow, "Isolation and characterization of a herpes simplex virus type 1 mutant containing a deletion within the gene encoding the immediate early polypeptide Vmw110," *Journal of General Virology*, vol. 67, no. 12, pp. 2571–2585, 1986.

[21] W. R. Sacks and P. A. Schaffer, "Deletion mutants in the gene encoding the herpes simplex virus type 1 immediate-early protein ICP0 exhibit impaired growth in cell culture," *Journal of Virology*, vol. 61, no. 3, pp. 829–839, 1987.

[22] F. Yao and P. A. Schaffer, "An activity specified by the osteosarcoma line U2OS can substitute functionally for ICP0, a major regulatory protein of herpes simplex virus type 1," *Journal of Virology*, vol. 69, no. 10, pp. 6249–6258, 1995.

[23] P. T. Sobol, J. L. Hummel, R. M. Rodrigues, and K. L. Mossman, "PML has a predictive role in tumor cell permissiveness to interferon-sensitive oncolytic viruses," *Gene Therapy*, vol. 16, no. 9, pp. 1077–1087, 2009.

[24] J. L. Hummel, E. Safroneeva, and K. L. Mossman, "The role of ICP0-Null HSV-1 and interferon signaling defects in the effective treatment of breast adenocarcinoma," *Molecular Therapy*, vol. 12, no. 6, pp. 1101–1110, 2005.

[25] G. Fulci, L. Breymann, D. Gianni et al., "Cyclophosphamide enhances glioma virotherapy by inhibiting innate immune responses," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 34, pp. 12873–12878, 2006.

[26] A. Friedman, J. P. Tian, G. Fulci, E. A. Chiocca, and J. Wang, "Glioma virotherapy: effects of innate immune suppression and increased viral replication capacity," *Cancer Research*, vol. 66, no. 4, pp. 2314–2319, 2006.

[27] O. Adams, K. Besken, C. Oberdörfer, C. R. MacKenzie, D. Rüßing, and W. Däubener, "Inhibition of human herpes simplex virus type 2 by interferon γ and tumor necrosis factor α is mediated by indoleamine 2,3-dioxygenase," *Microbes and Infection*, vol. 6, no. 9, pp. 806–812, 2004.

[28] D. H. Munn and A. L. Mellor, "Indoleamine 2,3-dioxygenase and tumor-induced tolerance," *Journal of Clinical Investigation*, vol. 117, no. 5, pp. 1147–1154, 2007.

[29] M. Zamanakou, A. E. Germenis, and V. Karanikas, "Tumor immune escape mediated by indoleamine 2,3-dioxygenase," *Immunology Letters*, vol. 111, no. 2, pp. 69–75, 2007.

[30] J. B. Katz, A. J. Muller, and G. C. Prendergast, "Indoleamine 2,3-dioxygenase in T-cell tolerance and tumoral immune escape," *Immunological Reviews*, vol. 222, no. 1, pp. 206–221, 2008.

[31] C. S. Hong, W. Fellows, A. Niranjan et al., "Ectopic matrix metalloproteinase-9 expression in human brain tumor cells enhances oncolytic HSV vector infection," *Gene Therapy*, vol. 17, no. 10, pp. 1200–1205, 2010.

[32] E. Tyminski, S. LeRoy, K. Terada et al., "Brain tumor oncolysis with replication-conditional herpes simplex virus type 1 expressing the prodrug-activating genes, CYP2B1 and secreted human intestinal carboxylesterase, in combination

with cyclophosphamide and irinotecan," *Cancer Research*, vol. 65, no. 15, pp. 6850–6857, 2005.

[33] J. M. Markert, P. G. Liechty, W. Wang et al., "Phase Ib trial of mutant herpes simplex virus G207 inoculated pre-and post-tumor resection for recurrent GBM," *Molecular Therapy*, vol. 17, no. 1, pp. 199–207, 2009.

[34] J. M. Markert, J. N. Parker, D. J. Buchsbaum, W. E. Grizzle, G. Y. Gillespie, and R. J. Whitley, "Oncolytic HSV-1 for the treatment of brain tumours," *Herpes*, vol. 13, no. 3, pp. 66–71, 2006.

[35] T. Mineta, S. D. Rabkin, T. Yazaki, W. D. Hunter, and R. L. Martuza, "Attenuated multi-mutated herpes simplex virus-1 for the treatment of malignant gliomas," *Nature Medicine*, vol. 1, no. 9, pp. 938–943, 1995.

[36] K. L. Mossman and J. R. Smiley, "Herpes simplex virus ICP0 and ICP34.5 counteract distinct interferon-induced barriers to virus replication," *Journal of Virology*, vol. 76, no. 4, pp. 1995–1998, 2002.

[37] W. R. Sacks and P. A. Schaffer, "Deletion mutants in the gene encoding the herpes simplex virus type 1 immediate-early protein ICP0 exhibit impaired growth in cell culture," *Journal of Virology*, vol. 61, no. 3, pp. 829–839, 1987.

[38] F. J. Jenkins and B. Roizman, "Herpes simplex virus 1 recombinants with noninverting genomes frozen in different isomeric arrangements are capable of independent replication," *Journal of Virology*, vol. 59, no. 2, pp. 494–499, 1986.

[39] R. J. Prestwich, F. Errington, R. M. Diaz et al., "The case of oncolytic viruses versus the immune system: waiting on the judgment of Solomon," *Human gene therapy*, vol. 20, no. 10, pp. 1119–1132, 2009.

[40] T. Miyazaki, K. Moritake, K. Yamada et al., "Indoleamine 2,3-dioxygenase as a new target for malignant glioma therapy: laboratory investigation," *Journal of Neurosurgery*, vol. 111, no. 2, pp. 230–237, 2009.

[41] G. J. Guillemin, K. M. Cullen, C. K. Lim et al., "Characterization of the kynurenine pathway in human neurons," *Journal of Neuroscience*, vol. 27, no. 47, pp. 12884–12892, 2007.

[42] F. Okano, W. J. Storkus, W. H. Chambers, I. F. Pollack, and H. Okada, "Identification of a novel HLA-A* 0201-restricted, cytotoxic T lymphocyte epitope in a human glioma-associated antigen, interleukin 13 receptor alpha2 chain," *Clinical Cancer Research*, vol. 8, pp. 2851–2855, 2002.

[43] M. R. Alvira, W. F. Goins, J. B. Cohen, and J. C. Glorioso, "Genetic studies exposing the splicing events involved in herpes simplex virus type 1 latency-associated transcript production during lytic and latent infection," *Journal of Virology*, vol. 73, no. 5, pp. 3866–3876, 1999.

[44] M. Tsvitov, A. R. Frampton, W. A. Shah et al., "Characterization of soluble glycoprotein D-mediated herpes simplex virus type 1 infection," *Virology*, vol. 360, no. 2, pp. 477–491, 2007.

[45] K. J. Livak and T. D. Schmittgen, "Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method," *Methods*, vol. 25, no. 4, pp. 402–408, 2001.

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Review Article

Structural Diversity in Conserved Regions Like the DRY-Motif among Viral 7TM Receptors—A Consequence of Evolutionary Pressure?

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Several herpes- and poxviruses have captured chemokine receptors from their hosts and modified these to their own benefit. The human and viral chemokine receptors belong to class A 7 transmembrane (TM) receptors which are characterized by several structural motifs like the DRY-motif in TM3 and the C-terminal tail. In the DRY-motif, the arginine residue serves important purposes by being directly involved in G protein coupling. Interestingly, among the viral receptors there is a greater diversity in the DRY-motif compared to their endogenous receptor homologous. The C-terminal receptor tail constitutes another regulatory region that through a number of phosphorylation sites is involved in signaling, desensitization, and internalization. Also this region is more variable among virus-encoded 7TM receptors compared to human class A receptors. In this review we will focus on these two structural motifs and discuss their role in viral 7TM receptor signaling compared to their endogenous counterparts.

1. Introduction

Seven transmembrane (7TM) receptors constitute the largest superfamily of membrane proteins and function as important mediators of extracellular signals to intracellular responses. The chemical diversity of the endogenous ligands is tremendous ranging from small simple chemical entities like photons, ions, and nucleotides, to more complex small ligands like monoamines and peptides, and larger proteins, glycoproteins, and lipids. The 7TM receptors are divided into five classes of which class A or rhodopsin-like receptors is the dominating class [1]. The receptors are characterized by seven membrane-spanning α-helices as well as coupling to G proteins; hence, the name is G protein coupled receptors (GPCRs). (In this review we will use the term 7TM receptors instead of GPCRs as these receptors also signal trough non-G protein-dependent pathways, like β-arrestin-mediated signaling [2].) Signaling by 7TM receptors through G proteins leads to, for example, either inhibition ($G_{\alpha i}$) or activation ($G_{\alpha s}$) of adenylyl cyclase and cAMP production, activation of phospholipase C with inositol triphosphate turnover ($G_{\alpha q}$), or activation of RhoGEF ($G_{\alpha 12/13}$) depending on which G protein the receptor is activating [3]. Furthermore, the $G_{\beta\gamma}$ subunit is also involved in signaling and the 7TM receptors also signal via G protein-independent pathways like MAP-kinase activation-mediated by β-arrestins [4].

Despite the structural diversity in the repertoire of the endogenous 7TM receptor agonists, the conformational changes that occur upon receptor activation are believed to be overall identical. Thus, as the last two decades of biochemical and biophysical studies indicate, TM6, and to a minor degree TM7 and TM3, undergo conformational rearrangement during receptor activation [5, 6]. Centered around the highly conserved proline in the middle of TM6 (position VI:15 or 6.50) TM6 is believed to perform movements that results in space creation thereby permitting binding

of intracellular signal transduction molecules like G proteins and β-arrestins [7]. (The numbering of amino acids in the helices is provided according to two numbering systems: the generic numbering system suggested by Schwartz [8], followed by the numbering system of Ballesteros and Weinstein [9],) Several crystal structures of 7TM receptors have been presented within the last decade initiated by the structure of bovine rhodopsin [10] followed by the adrenergic receptors [11–15], the adenosine receptors [16–18], additional rhodopsin variants [19–21], muscarinic receptors, [22, 23] and several others [24–26] including the chemokine receptor CXCR4 [27]. In the recent years, crystal structures of not only inactive, but also active 7TM receptors, have been identified. Thus, in the agonist-bound β2-adrenergic receptor, a relatively large rearrangement of the lower segments of TM6 is observed, when compared to the corresponding inactive structure [13–15]. This structural feature is also observed in the crystal structure of opsin in complex with a G protein peptide fragment upon comparison with dark-state rhodopsin [21, 28]. The overall arrangement of the seven transmembrane α-helices delineate the main binding pocket, and most studies in the search of functionally important residues have focused on amino acids facing this main binding pocket (delimited by TM3, TM4, TM5, TM6, and in part TM7). This is with good reason as most small molecule ligands interact with residues in this pocket [11, 12, 29]. Furthermore, most conserved microswitches of functional importance also face the main binding pocket. This includes ArgIII:26 (3.50), which is part of the conserved DRY-motif in TM3, the rotameric toggle switch TrpVI:13 (6.48), which is part of the CWxP-motif in TMVI, and TyrVII:20 (7.53), which is part the NPxxY-motif in TMVII—all of which play crucial roles during receptor activation [30, 31]. However, also residues in the region delimited by TM1, TM2, TM3, and TM7 (the so-called minor binding pocket) function as regulatory switches or major ligand anchor points [32–34].

The DRY-motif is the most conserved motif among the microswitches mentioned above (Figures 2(a) and 2(c)) [21, 30] and has been shown to directly interact with the G protein in a recent crystal structure of the β2-adrenergic receptor in complex with the $G_{\alpha s}$-subunit—a crystal that displayed the actual signaling complex and uncovered the importance of both the DRY-motif and the NPxxY-motif in receptor activation [14, 35]. While the overall interaction between the G protein and the receptor is mainly hydrophobic within the transmembrane core, the ArgIII:26 (3.50) is sandwiched between a Tyr in the G protein and TyrVI:20 (7.53) of the NPxxY-motif, highlighting the importance of concerted action of both motifs [14, 35].

The positively charged ArgIII:26 (3.50) has been proposed to be involved in other conformational constrains of importance for receptor activation. Thus, an inactivating salt bridge (a so-called ionic lock) has been suggested between the Arg and another conserved residue, the acidic GluVI:-05 (6.30) in intracellular loop 3 (ICL3) [36]. This ionic lock is broken during receptor activation where TyrVI:20 (7.53) rotates towards the helix bundle as seen in the active crystal structures of both rhodopsin [21] and the β2-adrenergic receptor irreversibly bound to an agonist [37] or stabilized by a nanobody [13]. However, as the GluVI:-05 (6.30) is only conserved among 25 % of all class A receptors [14, 35], and not present in any of the chemokine receptors [38], the molecular interactions involved in conformational constraining of inactive receptor states and the role of ArgIII:26 (3.50) must be different in receptors without GluVI:-05(6.30). Finally, the DRY-motif interacts with ICL2 of the receptor, thereby stabilizing a position of this loop capable of interacting with a hydrophobic pocket on the G protein and directly linking the highly conserved DRY-motif to the receptor/G protein interaction [14].

Another important region for receptor activity is the intracellular C-terminal tail of the 7TM receptors as it contains phosphorylation sites and other regulatory recognition motifs necessary for desensitization by G protein-coupled receptor kinases (GRKs), β-arrestin recruitment and signaling, internalization and receptor recycling, and for other means of signal regulation [39]. These two receptor motifs will be the focus of the current review, where we will compare the structural and functional properties, degree of conservation, and functional diversity of the two motifs between class A 7TM receptors encoded by viruses and endogenously encoded 7TM receptors. Most of the virus-encoded 7TM receptors belong to the chemokine subfamily [40] and consequently extra attention will be directed towards the viral molecular piracy within the chemokine system and the endogenous chemokine receptors.

2. The Chemokine System

The chemokine system plays an important role in the human immune defense against pathogens such as viruses since the chemokines (abbreviated from chemotactic cytokines) are involved in leukocyte migration during inflammation and also control activation and differentiation of lymphoid cells [41, 42]. The chemokine receptors belong to class A 7TM receptors and comprise the largest subfamily within this group with 19 different endogenous chemokine receptors and up to 50 chemokine ligands [43]. The chemokines are divided into four subfamilies depending on the presence or absence of residues between the first two of usually four conserved cysteines: the CXC (CXCL1-16) and CC (CCL1-28) chemokines along with the CX3C (CX3CL1) and XC (XCL1) chemokines [44, 45]. The CXC chemokines are further divided based on an ELR-motif prior to the CXC-motif. (In the following, the "novel" systematic chemokine nomenclature is used [44]) ELR CXC chemokines are induced under acute and chronic inflammation, play an angiogenic role, and mainly attract neutrophils while the non-ELR CXC chemokines exert their effect on lymphocytes and are more constitutively expressed as well as being angiostatic or angiomodulatory [46, 47]. The chemokine receptors are likewise divided into four groups in accordance with the classification of their preferred ligands [44, 48]. The interaction between chemokine and receptor range from high selectivity to large promiscuity; although not cross-interacting with other subfamilies. The main signaling

pathway of the endogenous chemokine receptors is via $G_{\alpha i}$ leading to calcium release and chemotaxis [49].

3. The Virus-Encoded 7TM Receptors

Considering the role of chemokines in the immune system it is not surprising that several viruses, by an act of molecular piracy of host genes, encode chemokines and/or chemokine receptors in their genomes. It is primarily the large poxviruses and the β- and γ-herpesviruses which encode chemokine receptors (and ligands) [50], however also the retrovirus HIV utilizes the endogenous chemokine system by using the two chemokine receptors CCR5 and CXCR4 as cell-entry co-factors together with CD4 during infection and spread [42, 51]. Also the viral CC (and CX3C) chemokine receptor homolog US28 (described further below) encoded by HCMV (human cytomegalovirus) has been implicated as a HIV cell-entry co-factor [52]. The majority of the viral receptors have structural features in common with the endogenous chemokine receptors in spite of having a sequence identity to these of only 25–59% [42]. However, compared to the endogenous chemokine receptors, the viral receptors show a vast divergence in their signaling capacities as well as ligand specificity with constitutive activity being typical for the viral receptors, unlike the endogenous chemokine receptors [53–57]. Constitutive activity also occurs among endogenous non-chemokine receptors, as shown for a few receptors [58]; however an increasing number of examples illustrate that the range of naturally-occurring constitutively activating mutations are tightly associated with disease or a particular phenotype [58]. This includes mutations in the melanocortin 1 receptor (MC1R; associated with melanism, [59]), MC4R (obesity, [60]), the ghrelin receptor (short stature, [61]) and rhodopsin (retinitis pigmentosa, [62]) among others.

Furthermore, besides being constitutively active, the viral receptors also signal promiscuously through many pathways, as compared to the predominant $G_{\alpha i}$ coupling of endogenous chemokine receptors [41, 47]. For instance the ORF74 (open reading frame 74) 7TM receptor encoded by HHV8 (human herpesvirus 8) associates with both $G_{\alpha i}$ and $G_{\alpha q}$ [63] as well as signals through MAP kinases [64] leading to the activation of numerous transcription factors, cell proliferation and transformation, VEGF secretion and angiogenesis [64–68]. The ORF74 from herpesvirus saimiri (HVS-ECRF3) also signals through both $G_{\alpha i}$, $G_{\alpha 12/13}$, and $G_{\alpha q}$ in a ligand-dependent manner, however the constitutive activity of this receptors is constrained to $G_{\alpha i}$ and $G_{\alpha 12/13}$, but not $G_{\alpha q}$ [69, 70]. A similar broad spectrum and promiscuous signaling is also observed for the US28 (unique short 28) and UL33 (unique long 33) 7TM receptors encoded by HCMV, which signals constitutively through both $G_{\alpha i}$, and $G_{\alpha q}$ along with MAP kinases [71–73].

Besides being evolutionary distinct from the endogenous chemokine receptors, the herpesvirus-encoded chemokine receptors cluster in four families (Figure 1): U12/UL33 of HHV6, HHV7, and CMV; U51/UL78 of HHV6, HHV7 and CMV; US27/US28 of CMV; and ORF74 of HHV8 as well as non-human herpesviruses [50]. (UL78 from CMV is evolutionarily conserved with U51 from HHV6 and HHV7. However, as the UL78 receptors have shown no functional homology to chemokine receptor, they have been excluded from current review.) The common feature of encoding chemokine receptors throughout the pox- and herpesviruses suggests that these receptors play an important role in the viral life cycle as well as in circumvention of the host immune system. A few studies of receptor-disrupted viruses have shown diminished replication *in vivo* in selected tissues [74, 75].

Chemokine receptors are also found in the genomes of poxviruses [76, 77]. In contrast to the broader subfamily resemblance to CC as well as CXC chemokine receptors along with the promiscuous chemokine-binding profile of many herpesvirus-encoded receptors, the poxvirus-encoded receptors solely resemble the CCR8 chemokine receptor (as illustrated in Figure 1) and only interact with CCR8-binding ligands [78, 79]. Briefly, the poxvirus-encoded receptors are located in two areas in the viral genome: 7L and 145R. The best characterized poxvirus receptors are 7L and 145R from YLDV (Yaba-like disease virus) [76, 77].

Also, nonchemokine receptors are found in viral genomes exemplified by the BILF family from several γ1-herpesviruses including human EBV (Epstein Barr virus). The BILF1 receptors from human and rhesus EBV are the only BILF receptors that have been characterized from a pharmacological point of view and like most other virus-encoded 7TM receptors they display constitutive activity [80, 81]. As an extra refinement and interplay with the host immune system, it should be mentioned that viruses also regulate the expression of endogenous 7TM receptors within the chemokine system, and class A in general. For instance, GPR183, also known as EBI2 (Epstein-Barr virus induced receptor 2), which is induced >200 fold upon EBV cell-entry [33, 40, 82].

4. The Impact of the DRY-Motif among Endogenous Class A Receptors

The Asp, Arg, Tyr, or DRY-motif in the intracellular end of TM3 is one of the most conserved motifs among class A 7TM receptors [31] (Figure 2(c)) and plays a pivotal role in receptor activation. This amino acid triplet is located in positions III:25 (3.49), III:26 (3.50), and III:27 (3.51), respectively, at the border to the intracellular loop 2 (ICL2) with a conservation as DRY in 66%, 96%, and 67% of all class A 7TM receptors [31]. The DRY-motif is even more conserved within the chemokine subfamily (Figure 2(a)) with 100% conservation of ArgIII:26 (3.50) and 95% conservation of both aromatic residues in position III:27 (3.51) and negatively charged residues in position III:25 (3.49).

As mentioned in the introduction, it has been suggested that, in some class A 7TM receptors (e.g., the β-adrenergic receptors and in rhodopsin) the ArgIII:26 (3.50) together with AspIII:25 (3.49) and GluVI:-05 (6.30), the latter located in ICL3, form an ionic lock holding TM3

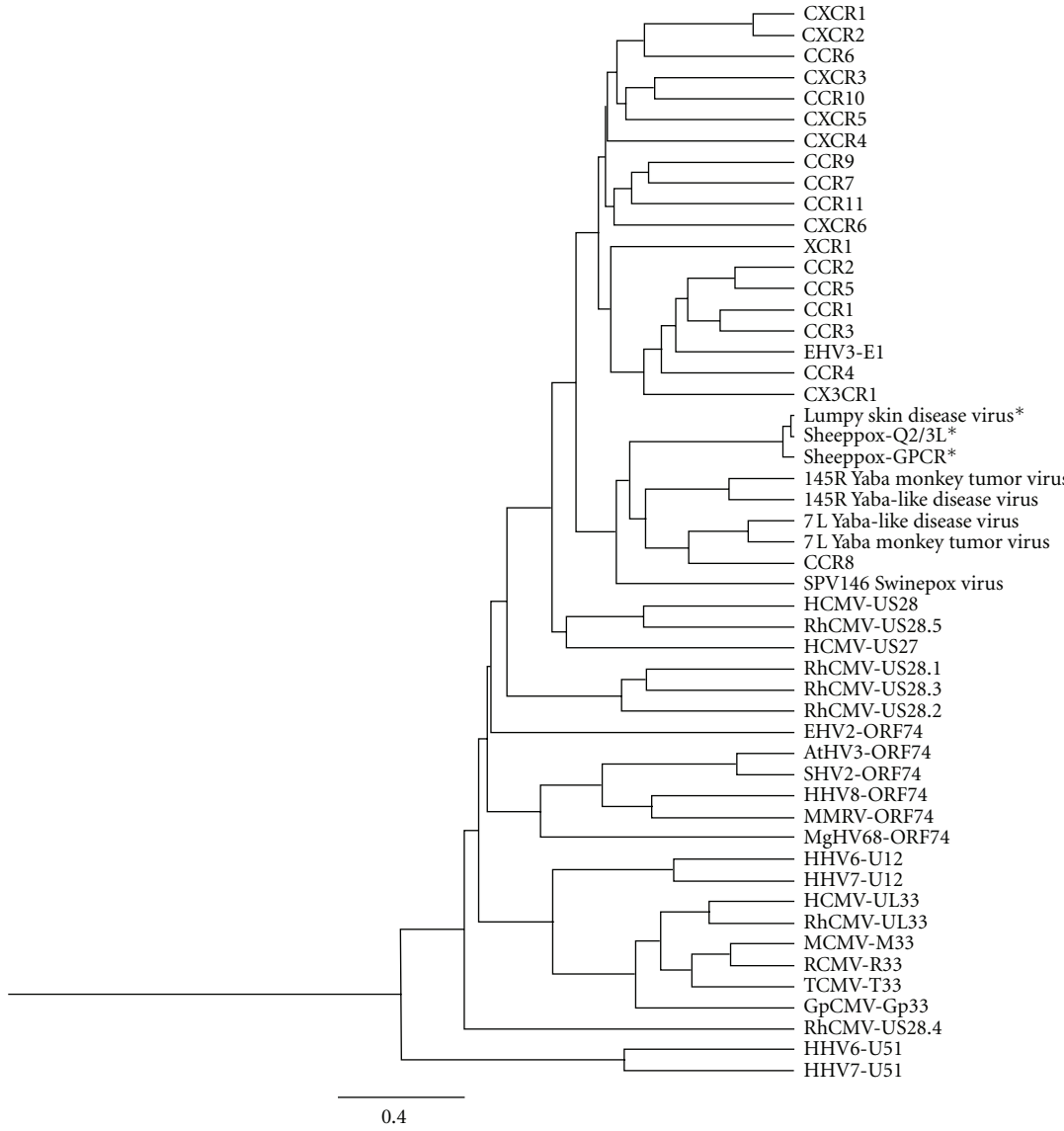

Figure 1: Phylogenetic tree of viral and human chemokine receptors based on their amino acid sequence. The length of each branch reflects the similarity between receptors. It was generated by aligning the sequences using the settings: Blosum62, gap open penalty of 5 and gap extension penalty of 0.1 followed by the Jukes-Cantor distance analysis done in Geneious Pro. For further information about the virus-encoded receptors and GenBank accession number please see Table 1. *As the sequence of these viral receptors are very alike it cannot be excluded that they are in fact the same.

and TM6 together in the inactive state [10, 36]. During receptor activation, protonation of AspIII:25 (3.49) leads to release of the constraining interaction, thus allowing the outward movement of TM6 [5, 83]. This is also supported by charged- neutralizing mutations of AspIII:25 (3.49) suggesting that this residue is important for receptor activation [84]. However, the negatively charged residue at position VI:-05 (6.30) is not nearly as conserved as the DRY-motif indicating other possible ways to constrain the receptor in the inactive conformation [30]. During receptor activation, the interaction between adjacent Asp/GluIII:25 (3.49) and ArgIII:26 (3.50) of the DRY-motif is disturbed and ArgIII:26 (3.50) is instead able to interact with TyrV:24 (5.58) as well as directly with the G_α protein. This direct interaction with the G protein has been confirmed by the crystal structure of Opsin in complex with a small peptide from the C-terminal of the $G_{\alpha t}$ protein [21]. Receptor activation opens a pocket at the intracellular site making the interaction with the C-terminal of the G_α protein possible. This allows for the exchange of GDP with GTP thus activating the G protein for further downstream signaling [85].

From the crystal structure of the chemokine receptor CXCR4, it is evident that the overall structure is similar to the other crystal structures of class A 7TM receptors, nevertheless with a few differences mainly in the extracellular part which constitutes the chemokine ligand-recognition

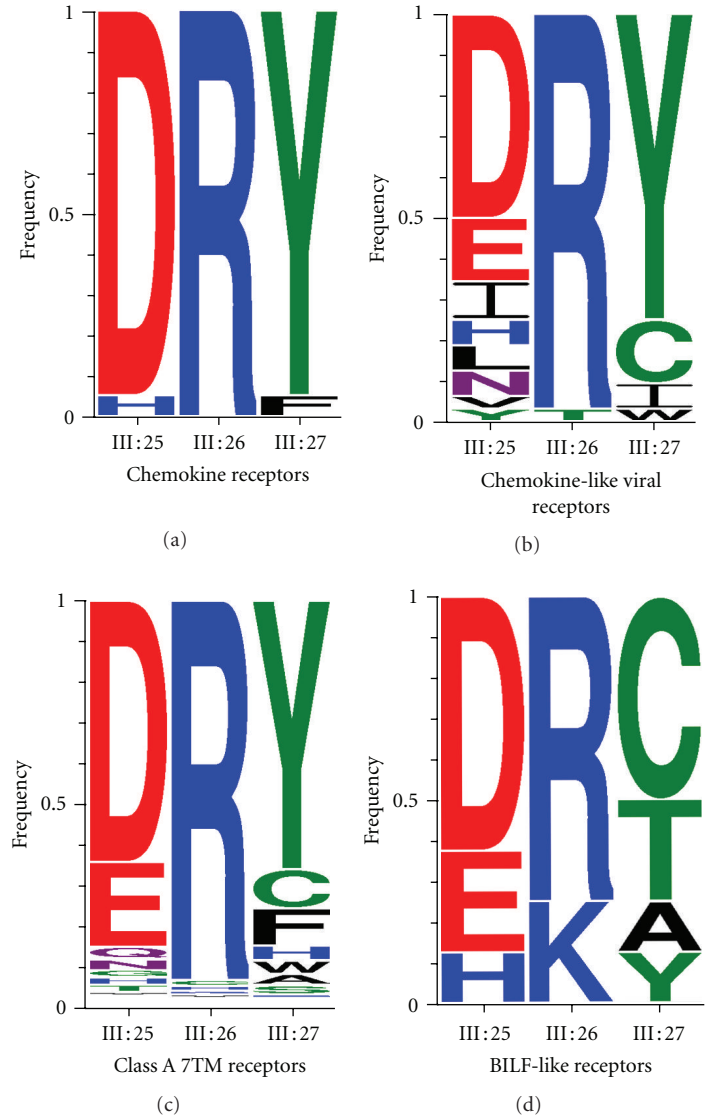

Figure 2: Sequence logos of the DRY-motif for chemokine receptors (a), chemokine-like viral receptors (b), class A 7TM receptors (c), and BILF-like receptors (d). The chemical properties of the amino acids are represented in color (polar: green, neutral: purple, basic: blue, acidic: red, and hydrophobic: black). This figure was created using the web application: http://weblogo.threeplusone.com/.

domain [27]. Importantly, the chemokine receptors (though belonging to class A) contain a *positively charged* residue at position VI:-05 (6.30) and hence the ionic lock between ArgIII:26 (3.50) and a negative charged residue at VI:-05 (6.30) does not exist in these receptors [38]. The lack of a negative charge in this position has inspired the introduction of the ionic lock in the chemokine receptors by substitution of the positive charge in VI:-05 (6.30) with a negative charge. This introduction of the putatively correct conditions for the ionic lock resulted in a reduced basal activity of the chemokine receptor CCR5, indicating that the receptor is locked in an inactive conformation with no ligand-induced activation and a strongly impaired ability to bind chemokines. Substitution of ArgIII:26 (3.50) with Ala or Gln maintained chemokine binding though still showed reduced basal activity [38]. Thus, the presence of an ionic lock between TM3 and TM6 leaves the chemokine receptor unable to switch to an active conformation; therefore, chemokine receptors must utilize a different mechanism than the classic ionic lock described above. However, the decrease in basal activity of CCR5 upon introduction of the ionic lock is in accordance with the general interpretation of the role of this motif in class A receptor activation, where loss of the ionic lock (VI:-05 (6.30) mutation), leads to constitutive activity [36]. Disregarding the lack of an ionic lock in chemokine receptors, the DRY-motif still plays an important role in the receptor activation, as exemplified in CCR5, where mutation of ArgIII:26 (3.50) to the neutral Asn disrupted both basal activity and chemokine-induced $G_{\alpha i}$ protein coupling (through calcium mobilization and GTPγS binding assays) despite retained affinity for CCL4 [86]. Interestingly, an increased basal phosphorylation of

Table 1

No.	Receptor	Accession number
1	Class A 7TM receptors	
2	CCR1	NP_001286.1
3	CCR2	NP_001116513.2
4	CCR3	NP_001828.1
5	CCR4	NP_005499.1
6	CCR5	NP_000570.1
7	CCR6	NP_004358.2
8	CCR7	NP_001829.1
9	CCR8	NP_005192.1
10	CCR9	NP_112477.1
11	CCR10	NP_057686.2
12	CCR11	NP_057641.1
13	CXCR1	NP_000625.1
14	CXCR2	NP_001548.1
15	CXCR3	NP_001495.1
16	CXCR4	NP_003458.1
17	CXCR5	NP_001707.1
18	CXCR6	NP_006555.1
19	CX3CR1	NP_001328.1
20	XCR1	NP_005274.1
21	HCMVUS28	P69332.1
22	RhCMVUS28.1	YP_068305.1
23	RhCMVUS28.2	AAN15199.1
24	RhCMVUS28.3	YP_068303.1
25	RhCMVUS28.4	YP_068302.1
26	RhCMVUS28.5	YP_068307.1
27	HCMVUS27	P09703.1
28	HCMVUL33	CAA37385.1
29	GpCMVGp33	AAK43591.1
30	RhCMVUL33	YP_068150.1
31	TCMVT33	NP_116383.1
32	MCMVM33	Q83207.1
33	RCMVR33	NP_064138.1
34	HHV6U12	P52380.1
35	HHV7U12	P52381.1
36	HHV6U51	NP_042944.1
37	HHV7U51	YP_073791.1
38	HHV8ORF74	AAC28486.1
39	MgHV68ORF74	NP_044914.1
40	AtHV3ORF74	NP_048046.1
41	SHV2ORF74	NP_040276.1
42	MMRVORF74	NP_570822.1
43	EHV2ORF74	NP_042670.1
44	EHV3E1	NP_042597.1
45	SPV 146 Swinepox virus	NP_570306.1
46	Sheeppox-GPCR	NP_659585.1
47	lumpy skin disease virus	AAN02735.1
48	Sheeppox-Q2/3L	Q86917.1
49	7L Yaba-like disease virus	NP_073392.1
50	7L Yaba Monkey Tumor virus	NP_938268.1
51	145R-Yaba Monkey Tumor virus	NP_938396.1
52	145R-Yaba-like disease virus	NP_073530.1

the ArgIII:26Asn- (3.50-) mutated receptor was observed along with β-arrestin-mediated endocytosis as well as a higher rate of internalization in response to CCL4 stimulation [86]. Other studies have indicated the need for an intact DRY-motif in β-arrestin1 binding to CCR5, thus supporting the importance of this motif for receptor function [87].

Another class A receptor lacking the ionic lock residue VI:-05 (6.30) is the histamine 4 receptor (H4R), which shows high constitutive activity. Introduction of GluVI:-05 (6.30) did, however, not decrease the constitutive activity [88] as expected from other studies where disruption of an already existing ionic lock leads to increased constitutive activity [36] or like the CCR5 chemokine receptor loss of activity upon ionic lock introduction [38]. Additionally, the H3R also shows constitutive activity in spite of having the putative conditions for an ionic lock [88–90] indicating that the histamine receptors have a functional difference from the general class A 7TM receptors when it comes to activation and constitutive activity. However, the H4R did show complete loss of G protein activation upon mutation of ArgIII:26 (3.50) supporting the importance of the DRY-motif for coupling to G proteins [85, 88, 91]. A similar phenomenon was observed in the H2R where charge-neutralizing mutations of ArgIII:26 (3.50) led to severely decreased basal cAMP production in terms of efficacy indicating diminished $G_{\alpha s}$ coupling. However, the mutated receptor was able to induce a response upon agonist stimulation though still with lower efficacy compared to wild type [92]. In the same study, it was shown that the charge-neutralizing mutations of ArgIII:26 also resulted in highly structurally instable receptors, where surface expression could only be detected after stabilization with either an agonist or inverse agonist, indicating a role not only in receptor activation, but also in receptor stability for this position [92]. Furthermore, the DRY-motif has also been implicated in receptor stability of the β2-adrenergic receptor [15].

The C5A binding protein, C5L2, is among the few 7TM receptors that lack a positive charge in position III:26, as it has a Leu in this position (together with Asp in III:25 (3.49) and Cys in III:27 (3.51) [93]). The native C5L2 is also known as a nonsignaling C5A binding protein, however, this impaired G protein coupling could be partially restored by reintroducing ArgIII:26 (3.50) [93]. Other endogenous class A receptors without a positive charge in III:26 (3.50) include D6 and Duffy antigen/receptor for chemokines (DARC)—two nonsignaling 7TM structured receptors belonging to the chemokine receptor system. Both are known as nonsignaling proteins as they do not couple to G proteins, but they exert chemokine scavenging and transendothelial transport instead. DARC is, furthermore, rather specifically expressed by endothelial cells lining postcapillary venules, and here it exerts its presumed role in accumulation of extravascular chemokines, chemokine transcytosis, and presentation on the luminal surface thereby facilitating leukocyte adhesion [94–97]. GPR77, GPR78, and GPR133 constitute three orphan class A receptors with unknown functions that also lack the ArgIII:26 (3.50), and, in addition a handful of receptors without positive, charge are identified among the olfactory receptors.

5. The DRY-Motif Is Less Conserved among Virus-Encoded 7TM Receptors

The conservation of the ArgIII:26 (3.50) in the DRY-motif is low among the virally encoded chemokine receptors when compared to the endogenous counterparts (Figures 2(a) and 2(b)). Furthermore, there is a much larger diversity with respect to all three residues as evident by Figure 2(b). These changes in the DRY-motif could be part of the reason for the altered signaling properties with higher constitutive activity, and activation of a broader range of signaling pathways [53, 98].

Regarding ArgIII:26 (3.50), one receptor deserves special notice, namely, the CXC chemokine receptor ORF74 from equine herpesvirus 2 (EHV2). This receptor contains a DTW-motif instead of the DRY consensus, thus missing a positive charged residue in position III:26 (3.50). In spite of this, the receptor shows constitutive activity through the $G_{\alpha i}$ pathway and ligand-mediated signaling in response to the endogenous chemokine CXCL6 [55, 99]. Interestingly, introduction of the DRY-motif in the EHV2-ORF74 led to a 4-fold decrease in constitutive activity while retaining activation by the agonist, CXCL6 [55], suggesting that this receptor has been optimized to act in the absence of a positive charge in the DRY-motif.

As is also evident from Figure 2(b), the virus-encoded chemokine receptors from different pox- and herpesviruses show a larger diversity in the whole DRY-motif; primarily with large deviations within the first residue III:25 (3.49). For instance, the CXC chemokine receptor ORF74 by HHV8 contains a VRY-motif in place of the endogenous DRY-motif. This receptor is associated with Kaposi's sarcoma and shows a high degree of constitutive signaling and stimulates proliferation [100] as well as tumor transformation in mice [64]. The closest endogenous chemokine receptor to HHV8-ORF74 is CXCR2, and studies have shown that replacement of AspIII:25 (3.49) with Val in CXCR2, thus making this receptor more ORF74-like with respect to this motif, leads to constitutive activation of CXCR2 with altered signaling properties in the direction of HHV8-ORF74 signaling [101]. In contrast, the opposite mutation in HHV8-ORF74 (ValIII:25Asp thereby reintroducing the DRY-motif) did not have major effects on either ligand binding or receptor signaling [102]. Another example is the ORF74 receptor from murine herpesvirus 68 (MHV68), which contains an HRC-motif and has been indicated to activate similar oncogenic pathways as HHV8-ORF74 [103, 104]. However, further studies are needed to determine the signaling capabilities of several ORF74 receptors as well as the influence of their altered DRY-motif on constitutive activity and regulatory circumvention.

The human herpesviruses HHV6 and HHV7 both encode two 7TM receptors, U12 and U51, which contain IRY- and ERI-motifs, respectively. HHV6-U12 has been shown to act as a chemokine receptor [105], whereas HHV6-U51 has been shown to be a constitutively active $G_{\alpha q}$-coupled CC chemokine receptor [106]. However, neither of these two receptors have the functional impact of the altered DRY-motif been studied.

The UL33 family, consisting of 7TM receptors from murine (M33), rat (R33), and human (UL33) cytomegalovirus, is known to constitutively signal through a vast array of G proteins [72]. The rodent counterparts differ from the human by containing an NRY-motif whereas UL33 contains the conserved DRY-motif. A mutational analysis of M33 revealed that ArgIII:26 (3.50) is important for the viral constitutive signaling in NFAT, CREB, and IP-turnover assays as mutation into a neutral Gln abolished constitutive activation of the receptor. The importance of this was supported by *in vivo* data where a virus with a missing ArgIII:26 (3.50), NRY changed to NQY, was unable to replicate in the salivary glands [107]. Very interestingly, mutation of AsnIII:25 (3.49) into the consensus AspIII:25 (3.49), NRY to DRY, leads to an increased constitutive signaling (especially through NFAT-mediated transcription) suggesting that the endogenous DRY-motif is preferable for high activity. Having *lower* receptor activity could be advantageous for the virus as this might be favorable for the virus life cycle [107]. In line with the results for M33, a mutation of ArgIII:26 (3.50) rendered the R33 receptor inactive with respect to G protein coupling [108]. However, in contrast to M33, it was found that replacement of AsnIII:25 (3.49) with the endogenous AspIII:25 (3.49) in R33 (NRY to DRY) did not change the constitutive activity, and that replacement with the nonpolar AlaIII:25 (3.49) (NRY to ARY) led to a diminished PLC stimulation, but an unaltered pertussis toxin-sensitive signaling indicating impaired $G_{\alpha q}$-signaling but maintained $G_{\alpha i}$.

Another HCMV-encoded receptor, US28, signals constitutively through several pathways such as $G_{\alpha q}$/phospholipase C, NFκB, CREB, and MAP kinases [73, 109, 110]. This viral chemokine receptor contains the conserved DRY-motif and a mutational analysis of ArgIII:26Ala found that disruption of the DRY-motif leads to impaired $G_{\alpha q}$ protein activation and IP-turnover in spite of wild-type levels of cell-surface expression [111]. Like HHV8-ORF74, US28 has been implicated in cancer as the constitutive signaling of the receptor can activate proliferative pathways leading to tumor formation [112–114]. Additionally, HCMV has been found in glioblastomas, which could indicate a possible role in tumorigenesis [115–118].

Also among the BILF receptors, found in several γ1-herpesviruses, has this motif obtained extra attention. Interestingly, the DRY-motif in the constitutively active BILF1 receptors from human and rhesus EBV differs from the consensus in all three residues being EKT (GluIII:25 (3.49), LysIII:26 (3.50), ThrIII:27 (3.51), Figure 2(d)) [80, 81]. Substitution of EKT with EAT in BILF1 from EBV resulted in abolished $G_{\alpha i}$ signaling, whereas the conservative substitution of Lys with Arg (ERT) signaled as wt BILF1 (EKT). Interestingly, introduction of the whole conserved motif (DRY) actually impaired the receptor activity partially, indicating that the EKT-motif is functionally superior to the conserved DRY-motif in this BILF1 receptor. In addition to the $G_{\alpha i}$ coupling, the authors tested the impact of the EAT-motif in NIH3T3 cell transformation and tumor growth in nude mice, and they found that also via these pathways the EAT-motif was completely silent compared to wt BILF1

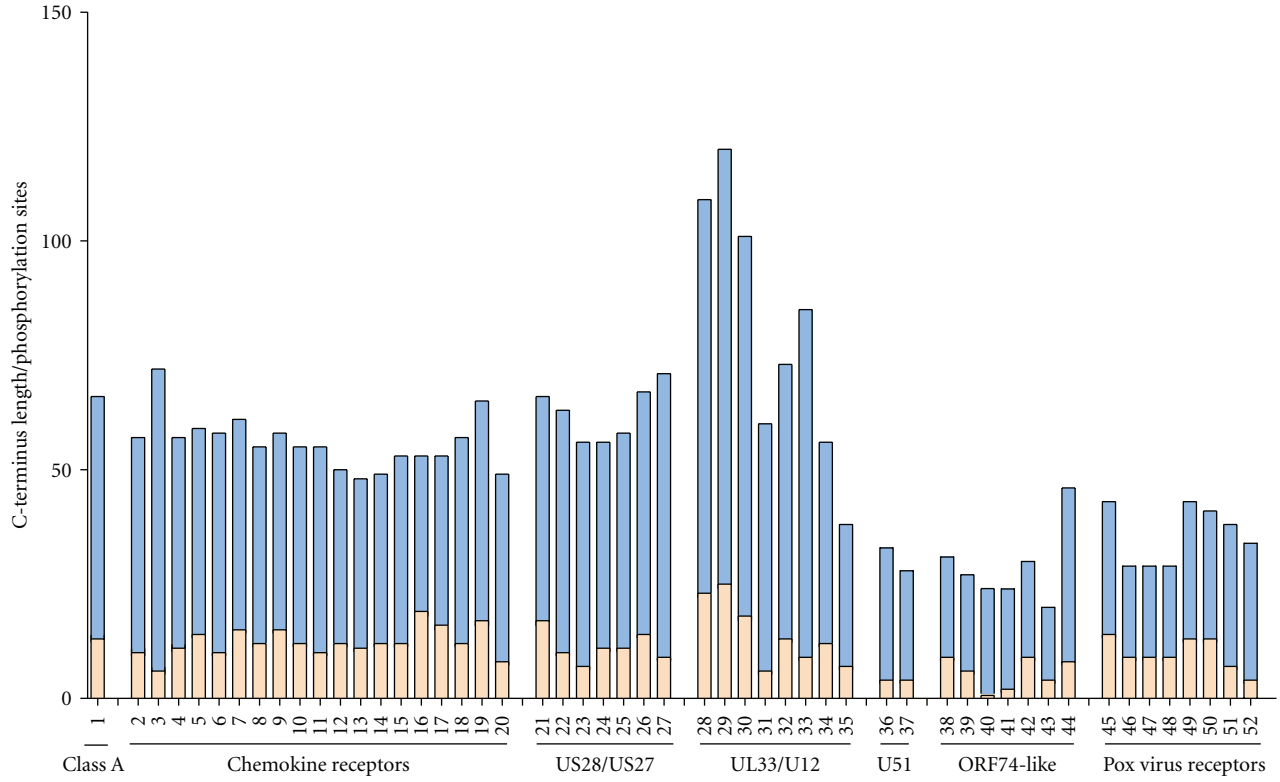

Figure 3: Relative sequence length and putative phosphorylation sites of the C-terminal region of class A 7TM receptors, chemokine receptors and virus-encoded chemokine receptors. The vertical axis displays the number of amino acids of the C-terminus (blue), defined as being after the highly conserved proline of the NPxxY-motif, and the number of serine, threonine and tyrosine in this region (beige). The horizontal axis displays the average of 334 non-olfactory class A 7TM receptors (1), the chemokine receptors (2–20) and the viral chemokine receptors (21–52). For further information about specific receptors and GenBank accession number please see Table 1.

(EKT). Furthermore, the DRY substitution displayed an intermediate active phenotype in these two functional readouts [119]. Thus, the BILF1 receptor depends on a positive charge in the DRY-motif and has, as a consequence of the altered motif (DRY to EKT), been optimized to signal with higher activity [80, 81, 119].

6. The C-Terminal Tails of Class A Receptors Are Conserved with Respect to Length and Number of Phosphorylation Sites

Whereas the extracellular N-terminal region of class A 7TM receptors are quite diverse, the intracellular C-terminal tails are more homologous, both in terms of length and primary structure. As evident from Figure 3, the average length and number of phosphorylation sites are similar among the endogenous chemokine receptors and class A 7TM receptors in general, whereas the virally encoded receptors show a larger diversity, but are generally shorter in length and have fewer phosphorylation sites. The phosphorylation sites serve important regulatory purposes for receptor desensitization and cell surface expression [120, 121]. Quickly after receptor activation and G protein interaction, GRKs initiate phosphorylation of serine and threonine residues in the C-terminal tail (and intracellular loops) thereby promoting the interaction of the receptor with β-arrestins and a subsequent steric hindering of the receptor/G protein-interaction [4]. The consequences of β-arrestin recruitment are endocytosis of the receptor/β-arrestin complex and a subsequent recycling to the cell surface or degradation. Interestingly, β-arrestins only associate with the ubiquitin ligase promoting the degradation pathway when it interacts with a ligand-stimulated receptor [122, 123]. The shorter C-terminal tails of the viral receptors could suggest that viruses circumvent the host regulatory processes of receptor internalization in order to obtain constitutive signaling abilities. Furthermore, some viral receptors are constitutively endocytosed and predominantly intracellularly localized [74, 124, 125], which have led to the suggestion, that they could act as scavengers (like DARC and D6 among endogenous chemokine receptors, see above) by internalizing the endogenous chemokine ligands that binds to the receptor and thereby removing the chemokines from the host cell surroundings as a way of evading the immune system, as discussed further below for the HCMV-encoded US28 [53, 124, 126, 127].

7. The C-Terminal Tail of Endogenous Chemokine Receptors

The endogenous chemokine receptors are rather similar in their C-terminal tails, and not different from the superfamily of endogenous class A 7TM receptors (Figure 3).

The 19 human chemokine receptors have in average the same number of phosphorylation sites as the endogenous class A 7TM receptors (13 in each case), and an average length of 57 residues, which is in the proximity of the 66 residues in average for the endogenous class A receptors. The internalization routes and regulation has been described for several endogenous chemokine receptors, an interest facilitated by the discovery of CCR5 and CXCR4 acting as HIV cell-entry cofactors [125, 128, 129]. Consequently, the endocytosis pattern of CCR5 and CXCR4 and the regulation of this have been studied in great detail and it was recently shown that internalization of CXCR4 plays an important antiviral role [130–133]. In the case of CCR5, the binding of CCL5 leads to receptor phosphorylation of serine residues in the C-terminal tail by GRKs, which consequently leads to internalization and desensitization of the signal [134, 135]. Besides the involvement of the serine residues in β-arrestin recruitment, a dileucine motif in the C-terminal tail is also important for CCR5 receptor endocytosis [136]. Serial truncation of the CCR5 C-terminal tail resulted in progressive loss of cell surface expression, which could not be rescued by substitution with the C-terminal tail of CXCR4 [137]. Mutational analysis of CXCR4 showed that this receptor is likewise dependent on C-terminal serine phosphorylation sites and a dileucine motif for proper receptor internalization [138, 139]. Internalization of CXCR4 can follow two distinct pathways: CXCL12 ligand-mediated endocytosis was shown to be dependent on the serine phosphorylation sites whereas phorbol ester induced internalization is dependent on the dileucine motif [131, 132]. Interestingly, a naturally occurring mutation of CXCR4 with C-terminal deletions exists in patients with WHIM syndrome (warts, hypogammaglobulinemia, recurrent bacterial infection, myelokathexis). Loss of the C-terminal tail leads to decreased endocytosis of the receptor and consequently a reduced regulation of the receptor followed by an increased signaling with enhanced calcium flux and cell migration; a possible cause of the pathophysiology seen in WHIM syndrome [140]. Thus, the C-terminal tail plays an important role in the physiology of endogenous chemokine receptors.

8. The C-Terminal Tails of Virus-Encoded Receptors Are Generally Shorter

The HCMV-encoded chemokine receptor US28 signals constitutively via several pathways and upon stimulation by CC chemokines [54, 73]. Furthermore, CX3CL1 has been reported to act as an inverse agonist, albeit with low efficacy (up to 25% inhibition of basal activity) [141]. Additionally, unlike the majority of endogenous class A 7TM receptors, US28 is constitutively internalized in a ligand-independent manner [126, 127, 142]. Thus, by immunofluorescence staining, US28 was found to be accumulated intracellularly in endocytic organelles and by advanced immunogold electron microscopy shown to be localized to multivesicular endosomes [126]. Further studies revealed that US28 endocytosis occurs via a clathrin-mediated mechanism [127]. Importantly, only a small fraction of US28 is present at the cell surface (<20%), with the rest undergoing constitutive ligand-independent endocytosis with a fast internalization rate, as compared to CXCR4. Truncation of the C-terminal tail of US28 led to an increase in both magnitude and duration of the constitutive signaling indicating that the C-terminal tail plays a regulatory role in desensitizing the receptor. This was supported by hyperactivation of US28 in cells where β-arrestin 1 and 2 were genetically deleted [141]. A mutational analysis of serine residues in the C-terminal of US28 revealed that a decreased number of phosphorylation sites increased the cell surface expression of the receptor [143]. Truncating the C-terminal tail of US28 or replacing it with tails from other 7TM receptors (HHV8-ORF74 and human tachykinin NK1) led to an increase in constitutive activity of the receptor. Substitution of the HHV8-ORF74 tail with the tail from US28 diminished the cell surface expression of the HHV8-ORF74 chimera indicating that the C-terminal tail, in itself, is sufficient for desensitization by receptor endocytosis [144]. The constitutive endocytosis of US28 may serve as a chemokine scavenger and mediate the viral immune evasion by antagonizing the recruitment of cells involved in the immune response and thereby manipulating the host immune system. Another HCMV-encoded chemokine receptor, US27, also shows a large degree of intracellular localization. Swapping the C-terminal tail of US27 with that of the endogenous chemokine receptor CXCR3 led to cell surface expression similar to wild-type CXCR3; likewise, when substituting the endogenous tail with the viral US27 tail, the chimeric receptor was predominantly located intracellularly indicating that the C-terminal tail of US27 is necessary and sufficient for intracellular localization [145].

In general, the viral chemokine receptors, ORF74, encoded by several herpesviruses have very short C-terminal tails when compared to the endogenous receptors (Figure 3). A study of several γ-herpesviruses identified an eight-amino-acid conserved region at the membrane proximal part of the C-terminal tail suggested to play a role in G protein coupling and G_α-selectivity [146]. Especially one basic residue showed importance for $G_{\alpha q}$ coupling—a residue which is conserved among the endogenous chemokine receptors suggesting an evolutionary conserved function of this residue such as G protein signaling [146]. HHV8-ORF74 is primarily located at the cell surface and deletion of the five terminal amino acids containing 3 phosphorylation sites did not seem to affect cell surface expression, though it did impact the signaling capabilities of the receptor seen by a diminished NFκB and AP-1 signaling [147]. As signaling deficiencies are seen by the removal of just five amino acids, it is tempting to consider that the length of the C-terminal tail has been optimized to only contain necessities and thus demonstrating a minimum requirement for a functional viral receptor tail. Another study also found expression levels of 12 and 24 amino acids C-terminal tail deletions similar to wild-type albeit with reduced constitutive activity in spite of retained ligand regulation by chemokines [148]. It was suggested that the C-terminal helix 8, which is present in the terminal 24 amino acids, is involved in stabilizing the interaction between the receptor and G protein, thus playing a role in mediating

Table 2

No.	Receptor	Accession number
1	Class A 7TM receptors	
2	CaHV3-ORF6	NP_733858.1
3	CHV15-BILF1-rh	YP_068006.1
4	EBV-BILF1	YP_401711.1
5	EHV2-E6	NP_042607.1
6	AlceHV-E5	NP_065513.1
7	PLHV3-A5	AAO12316.1
8	PHV2-A5	AAF16523.1
9	PLHV1-A5	AAF16521.1

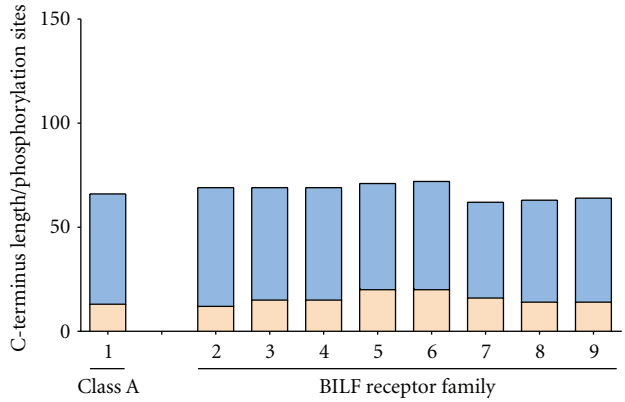

Figure 4: Relative sequence length and putative phosphorylation sites of the C-terminal region of class A 7TM receptors and the BILF-like receptors. The vertical axis displays the number of amino acids of the C-terminus (blue), defined as being after the highly conserved proline of the NPxxY-motif (class A 7TM receptors) or predicted by the Transmembrane Hidden Markov model using Geneious Pro (BILF receptor family), and the number of serine, threonine, and tyrosine in this region (beige). The horizontal axis displays the average of 334 non-olfactory class A 7TM receptors (1) and the EBV-encoded BILF receptors (2–9). For further details about specific receptors and GenBank accession number please see Table 2.

signals upon chemokine binding [148]. Though the C-terminal tail of ORF74 appears to be involved in signal mediation, deletion of small parts of the tail including phosphorylation sites does not seem to largely affect cell surface expression or constitutive signaling suggesting that a short tail is enough for the receptor to function to a certain degree. Having such short tails might be a way of evading host regulatory mechanisms, such as GRKs and internalization, thereby ensuring virus-mediated constitutive signaling.

The BILF receptor family is rather conserved when it comes to the length of their C-terminal tails suggesting that the C-terminus serves an important purpose for the virus (Figure 4). The BILF1 receptor encoded by EBV shows a similar cell surface expression pattern as HHV8-ORF74 and signal constitutively through $G_{\alpha i}$ [80]. Though the BILF1 receptor does not resemble the endogenous chemokine receptors, it does serve a purpose in viral immune evasion as it is involved in internalization and degradation of MHC-I (major histocompatibility complex class I) molecules. Deletion of the C-terminal tail of the receptor led to impaired lysosomal degradation of internalized MHC-I molecules suggesting that the tail might contain a localization sequence guiding the receptor/MHC-I complex to the lysosomes [149].

9. Summary

From what is reviewed above, it is evident that the virus-encoded 7TM receptors differ from the endogenous counterparts—both from a structural and a functional point of view. The viral receptors have been captured from the host and through evolution (i.e., combinatorial chemistry by random mutagenesis followed by natural selection of the most virulent strain) been optimized to benefit the virus life cycle. As the chemokine receptor exploitation (and the general 7TM receptor piracy) is a widespread phenomenon among many viruses, it is likely that these receptors serve important purposes for virus survival, for instance, evasion of the antimicrobial immune response, viral persistence, viral dissemination, and control of own infection as shown for a few receptors. The selection of the chemokine system for interference by the viruses points towards this system as essential in multiple different immune responses. By studying the structural and functional alterations in the virus-encoded receptors as compared to the endogenous receptors, greater knowledge can be obtained for 7TM receptors in general. Thus, from a molecular pharmacology point of view, the chemokine receptors represent unique opportunities to study basic principles of receptor activation, internalization, and recycling pathways as examples of targeted evolution where the receptors have undergone major changes driven by a heavy evolutionary pressure. Since 7TM receptors are excellent drug targets, the development of high-potency antagonists or inverse agonists for the virus-encoded 7TM receptors could putatively pave the path for tomorrow's antiviral and anti-inflammatory drugs.

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References

[1] D. M. Rosenbaum, S. G. F. Rasmussen, and B. K. Kobilka, "The structure and function of G-protein-coupled receptors," *Nature*, vol. 459, no. 7245, pp. 356–363, 2009.

[2] K. Rajagopal, R. J. Lefkowitz, and H. A. Rockman, "When 7 transmembrane receptors are not G protein-coupled receptors," *Journal of Clinical Investigation*, vol. 115, no. 11, pp. 2971–2974, 2005.

[3] T. M. Cabrera-Vera et al., "Insights into G protein structure, function, and regulation," *Endocrine Reviews*, vol. 24, no. 6, pp. 765–781, 2003.

[4] R. J. Lefkowitz, "Seven transmembrane receptors: something old, something new," *Acta Physiologica*, vol. 190, no. 1, pp. 9–19, 2007.

[5] C. Altenbach, A. K. Kusnetzow, O. P. Ernst, K. P. Hofmann, and W. L. Hubbell, "High-resolution distance mapping in rhodopsin reveals the pattern of helix movement due to activation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 21, pp. 7439–7444, 2008.

[6] W. L. Hubbell, C. Altenbach, C. M. Hubbell, and H. G. Khorana, "Rhodopsin structure, dynamics, and activation: a perspective from crystallography, site-directed spin labeling, sulfhydryl reactivity, and disulfide cross-linking," *Advances in Protein Chemistry*, vol. 63, pp. 243–290, 2003.

[7] T. W. Schwartz, T. M. Frimurer, B. Holst, M. M. Rosenkilde, and C. E. Elling, "Molecular mechanism of 7tm receptor activation—a global toggle switch model," *Annual Review of Pharmacology and Toxicology*, vol. 46, pp. 481–519, 2006.

[8] T. W. Schwartz, "Locating ligand-binding sites in 7tm receptors by protein engineering," *Current Opinion in Biotechnology*, vol. 5, no. 4, pp. 434–444, 1994.

[9] J. A. Ballesteros and H. Weinstein, "Integrated methods for the construction of three-dimensional models and computational probing of structure-function relations in G protein-coupled receptors," in *Receptor molecular biology*, S. C. Sealfon, Ed., Academic Press, New York, NY, USA, 1995.

[10] K. Palczewski, T. Kumasaka, T. Hori et al., "Crystal structure of rhodopsin: a G protein-coupled receptor," *Science*, vol. 289, no. 5480, pp. 739–745, 2000.

[11] V. Cherezov, D. M. Rosenbaum, M. A. Hanson et al., "High-resolution crystal structure of an engineered human β2-adrenergic G protein-coupled receptor," *Science*, vol. 318, no. 5854, pp. 1258–1265, 2007.

[12] T. Warne et al., "Structure of a beta1-adrenergic G-protein-coupled receptor," *Nature*, no. 7203, pp. 486–491, 2008.

[13] S. G. F. Rasmussen, H. J. Choi, J. J. Fung et al., "Structure of a nanobody-stabilized active state of the β2 adrenoceptor," *Nature*, vol. 469, no. 7329, pp. 175–180, 2011.

[14] S. G. Rasmussen et al., "Crystal structure of the beta 2 adrenergic receptor-Gs protein complex," *Nature*, vol. 477, no. 7366, pp. 549–555, 2011.

[15] S. G. Rasmussen et al., "Crystal structure of the human beta2 adrenergic G-protein-coupled receptor," *Nature*, vol. 450, no. 7168, pp. 383–387, 2007.

[16] A. S. Dore et al., "Structure of the adenosine A(2A) receptor in complex with ZM241385 and the xanthines XAC and caffeine," *Structure*, vol. 19, no. 9, pp. 1283–1293, 2011.

[17] G. Lebon, T. Warne, P. C. Edwards et al., "Agonist-bound adenosine A2A receptor structures reveal common features of GPCR activation," *Nature*, vol. 474, no. 7352, pp. 521–525, 2011.

[18] F. Xu, H. Wu, V. Katritch et al., "Structure of an agonist-bound human A2A adenosine receptor," *Science*, vol. 332, no. 6027, pp. 322–327, 2011.

[19] H. W. Choe, Y. J. Kim, J. H. Park et al., "Crystal structure of metarhodopsin II," *Nature*, vol. 471, no. 7340, pp. 651–655, 2011.

[20] J. Standfuss, P. C. Edwards, A. D'Antona et al., "The structural basis of agonist-induced activation in constitutively active rhodopsin," *Nature*, vol. 471, no. 7340, pp. 656–660, 2011.

[21] P. Scheerer, J. H. Park, P. W. Hildebrand et al., "Crystal structure of opsin in its G-protein-interacting conformation," *Nature*, vol. 455, no. 7212, pp. 497–502, 2008.

[22] K. Haga, A. C. Kruse, H. Asada et al., "Structure of the human M2 muscarinic acetylcholine receptor bound to an antagonist," *Nature*, vol. 482, no. 7386, pp. 547–551, 2012.

[23] A. C. Kruse, J. Hu, A. C. Pan et al., "Structure and dynamics of the M3 muscarinic acetylcholine receptor," *Nature*, vol. 482, no. 7386, pp. 552–556, 2012.

[24] E. Y. T. Chien, W. Liu, Q. Zhao et al., "Structure of the human dopamine D3 receptor in complex with a D2/D3 selective antagonist," *Science*, vol. 330, no. 6007, pp. 1091–1095, 2010.

[25] M. A. Hanson, C. B. Roth, E. Jo et al., "Crystal structure of a lipid G protein-coupled receptor," *Science*, vol. 335, no. 6070, pp. 851–855, 2012.

[26] T. Shimamura et al., "Structure of the human histamine H1 receptor complex with doxepin," *Nature*, vol. 475, no. 7354, pp. 65–70, 2011.

[27] B. Wu, E. Y. T. Chien, C. D. Mol et al., "Structures of the CXCR4 chemokine GPCR with small-molecule and cyclic peptide antagonists," *Science*, vol. 330, no. 6007, pp. 1066–1071, 2010.

[28] J. H. Park, P. Scheerer, K. P. Hofmann, H. W. Choe, and O. P. Ernst, "Crystal structure of the ligand-free G-protein-coupled receptor opsin," *Nature*, vol. 454, no. 7201, pp. 183–187, 2008.

[29] V. P. Jaakola, M. T. Griffith, M. A. Hanson et al., "The 2.6 angstrom crystal structure of a human A2A adenosine receptor bound to an antagonist," *Science*, vol. 322, no. 5905, pp. 1211–1217, 2008.

[30] R. Nygaard, T. M. Frimurer, B. Holst, M. M. Rosenkilde, and T. W. Schwartz, "Ligand binding and micro-switches in 7tm receptor structures," *Trends in Pharmacological Sciences*, vol. 30, no. 5, pp. 249–259, 2009.

[31] T. Mirzadegan, G. Benko, S. Filipek, and K. Palczewski, "Sequence analyses of G-protein-coupled receptors: similarities to rhodopsin," *Biochemistry*, vol. 42, no. 10, pp. 2759–2767, 2003.

[32] M. M. Rosenkilde, T. Benned-Jensen, T. M. Frimurer, and T. W. Schwartz, "The minor binding pocket: a major player in 7tm receptor activation," *Trends in Pharmacological Sciences*, vol. 31, no. 12, pp. 567–574, 2010.

[33] T. Benned-Jensen and M. M. Rosenkilde, "Structural motifs of importance for the constitutive activity of the orphan 7tm receptor EBI2: analysis of receptor activation in the absence of an agonist," *Molecular Pharmacology*, vol. 74, no. 4, pp. 1008–1021, 2008.

[34] T. Benned-Jensen and M. M. Rosenkilde, "The role of transmembrane segment II in 7tm receptor activation," *Current Molecular Pharmacology*, vol. 2, no. 2, pp. 140–148, 2009.

[35] T. W. Schwartz and T. P. Sakmar, "Structural biology: snapshot of a signalling complex," *Nature*, vol. 477, no. 7366, pp. 540–541, 2011.

[36] J. A. Ballesteros, A. D. Jensen, G. Liapakis et al., "Activation of the beta 2-adrenergic receptor involves disruption of an ionic lock between the cytoplasmic ends of transmembrane segments 3 and 6," *Journal of Biological Chemistry*, vol. 276, no. 31, pp. 29171–29177, 2001.

[37] D. M. Rosenbaum, C. Zhang, J. A. Lyons et al., "Structure and function of an irreversible agonist-β2 adrenoceptor complex," *Nature*, vol. 469, no. 7329, pp. 236–240, 2011.

[38] J. Y. Springael, C. de Poorter, X. Deupi, J. Van Durme, L. Pardo, and M. Parmentier, "The activation mechanism of chemokine receptor CCR5 involves common structural changes but a different network of interhelical interactions

relative to rhodopsin," *Cellular Signalling*, vol. 19, no. 7, pp. 1446–1456, 2007.

[39] K. N. Nobles, K. Xiao, S. Ahn et al., "Distinct phosphorylation sites on the β_2-adrenergic receptor establish a barcode that encodes differential functions of β-arrestin," *Science Signaling*, vol. 4, no. 185, Article ID ra51, 2011.

[40] M. M. Rosenkilde and N. Kledal, "Targeting herpesvirus reliance of the chemokine system," *Current Drug Targets*, vol. 7, no. 1, pp. 103–118, 2006.

[41] M. M. Rosenkilde, "Virus-encoded chemokine receptors—putative novel antiviral drug targets," *Neuropharmacology*, vol. 48, no. 1, pp. 1–13, 2005.

[42] P. M. Murphy, "Viral exploitation and subversion of the immune system through chemokine mimicry," *Nature Immunology*, vol. 2, no. 2, pp. 116–122, 2001.

[43] M. M. Rosenkilde and T. W. Schwartz, "Glu VII:06—a highly conserved and selective anchor point for non-peptide ligands in chemokine receptors," *Current Topics in Medicinal Chemistry*, vol. 6, no. 13, pp. 1319–1333, 2006.

[44] P. M. Murphy, M. Baggiolini, I. F. Charo et al., "International union of pharmacology. XXII. Nomenclature for chemokine receptors," *Pharmacological Reviews*, vol. 52, no. 1, pp. 145–176, 2000.

[45] P. M. Murphy, "International union of pharmacology. XXX. Update on chemokine receptor nomenclature," *Pharmacological Reviews*, vol. 54, no. 2, pp. 227–229, 2002.

[46] R. M. Strieter, P. J. Polverini, S. L. Kunkel et al., "The functional role of the ELR motif in CXC chemokine-mediated angiogenesis," *Journal of Biological Chemistry*, vol. 270, no. 45, pp. 27348–27357, 1995.

[47] M. M. Rosenkilde and T. W. Schwartz, "The chemokine system—a major regulator of angiogenesis in health and disease," *APMIS*, vol. 112, no. 7-8, pp. 481–495, 2004.

[48] P. J. Holst and M. M. Rosenkilde, "Microbiological exploitation of the chemokine system," *Microbes and Infection*, vol. 5, no. 2, pp. 179–187, 2003.

[49] E. R. Neptune and H. R. Bourne, "Receptors induce chemotaxis by releasing the $\beta\gamma$ subunit of Gi, not by activating Gq or Gs," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 26, pp. 14489–14494, 1997.

[50] M. M. Rosenkilde, M. Waldhoer, H. R. Lüttichau, and T. W. Schwartz, "Virally encoded 7tm receptors," *Oncogene*, vol. 20, no. 13, pp. 1582–1593, 2001.

[51] H. Choe, K. A. Martin, M. Farzan, J. Sodroski, N. P. Gerard, and C. Gerard, "Structural interactions between chemokine receptors, gp120 Env and CD4," *Seminars in Immunology*, vol. 10, no. 3, pp. 249–257, 1998.

[52] O. Pleskoff, C. Tréboute, A. Brelot, N. Heveker, M. Seman, and M. Alizon, "Identification of a chemokine receptor encoded by human cytomegalovirus as a cofactor for HIV-1 entry," *Science*, vol. 276, no. 5320, pp. 1874–1878, 1997.

[53] M. M. Rosenkilde, M. J. Smit, and M. Waldhoer, "Structure, function and physiological consequences of virally encoded chemokine seven transmembrane receptors," *British Journal of Pharmacology*, vol. 153, supplement 1, pp. S154–S166, 2008.

[54] M. Waldhoer, T. N. Kledal, H. Farrell, and T. W. Schwartz, "Murine cytomegalovirus (CMV) M33 and human CMV US28 receptors exhibit similar constitutive signaling activities," *Journal of Virology*, vol. 76, no. 16, pp. 8161–8168, 2002.

[55] M. M. Rosenkilde, T. N. Kledal, and T. W. Schwartz, "High constitutive activity of a virus-encoded seven transmembrane receptor in the absence of the conserved DRY motif (Asp-Arg-Tyr) in transmembrane helix 3," *Molecular Pharmacology*, vol. 68, no. 1, pp. 11–19, 2005.

[56] M. J. Smit, D. Verzijl, P. Casarosa, M. Navis, H. Timmerman, and R. Leurs, "Kaposi's sarcoma-associated herpesvirus-encoded G protein-coupled receptor ORF74 constitutively activates p44/p42 MAPK and Akt via Gi and phospholipase C-dependent signaling pathways," *Journal of Virology*, vol. 76, no. 4, pp. 1744–1752, 2002.

[57] K. A. McLean, P. J. Holst, L. Martini, T. W. Schwartz, and M. M. Rosenkilde, "Similar activation of signal transduction pathways by the herpesvirus-encoded chemokine receptors US28 and ORF74," *Virology*, vol. 325, no. 2, pp. 241–251, 2004.

[58] R. Seifert and K. Wenzel-Seifert, "Constitutive activity of G-proteins-coupled receptors: cause of disease and common property of wild-type receptors," *Naunyn-Schmiedeberg's Archives of Pharmacology*, vol. 366, no. 5, pp. 381–416, 2002.

[59] L. S. Robbins, J. H. Nadeau, K. R. Johnson et al., "Pigmentation phenotypes of variant extension locus alleles result from point mutations that alter MSH receptor function," *Cell*, vol. 72, no. 6, pp. 827–834, 1993.

[60] S. Srinivasan, C. Lubrano-Berthelier, C. Govaerts et al., "Constitutive activity of the melanocortin-4 receptor is maintained by its N-terminal domain and plays a role in energy homeostasis in humans," *Journal of Clinical Investigation*, vol. 114, no. 8, pp. 1158–1164, 2004.

[61] J. Pantel, M. Legendre, S. Cabrol et al., "Loss of constitutive activity of the growth hormone secretagogue receptor in familial short stature," *Journal of Clinical Investigation*, vol. 116, no. 3, pp. 760–768, 2006.

[62] P. R. Robinson, G. B. Cohen, E. A. Zhukovsky, and D. D. Oprian, "Constitutively active mutants of rhodopsin," *Neuron*, vol. 9, no. 4, pp. 719–725, 1992.

[63] M. M. Rosenkilde, T. N. Kledal, H. Bräuner-Osborne, and T. W. Schwartz, "Agonists and inverse agonists for the herpesvirus 8-encoded constitutively active seven-transmembrane oncogene product, ORF-74," *Journal of Biological Chemistry*, vol. 274, no. 2, pp. 956–961, 1999.

[64] C. Bais, B. Santomasso, O. Coso et al., "G-protein-coupled receptor of Kaposi's sarcoma-associated herpesvirus is a viral oncogene and angiogenesis activator," *Nature*, vol. 391, no. 6662, pp. 86–89, 1998.

[65] A. Sodhi, S. Montaner, V. Patel et al., "The Kaposi's sarcoma-associated herpes virus G protein-coupled receptor up-regulates vascular endothelial growth factor expression and secretion through mitogen-activated protein kinase and p38 pathways acting on hypoxia-inducible factor 1α," *Cancer Research*, vol. 60, no. 17, pp. 4873–4880, 2000.

[66] S. Montaner, A. Sodhi, S. Pece, E. A. Mesri, and J. S. Gutkind, "The Kaposi's sarcoma-associated herpesvirus G protein-coupled receptor promotes endothelial cell survival through the activation of Akt/protein kinase B," *Cancer Research*, vol. 61, no. 6, pp. 2641–2648, 2001.

[67] S. Montaner, A. Sodhi, A. Molinolo et al., "Endothelial infection with KSHV genes in vivo reveals that vGPCR initiates Kaposi's sarcomagenesis and can promote the tumorigenic potential of viral latent genes," *Cancer Cell*, vol. 3, no. 1, pp. 23–36, 2003.

[68] A. Sodhi, S. Montaner, V. Patel et al., "Akt plays a central role in sarcomagenesis induced by Kaposi's sarcoma herpesvirus-encoded G protein-coupled receptor," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 14, pp. 4821–4826, 2004.

[69] M. M. Rosenkildel, K. A. McLean, P. J. Holst, and T. W. Schwartz, "The CXC chemokine receptor encoded by herpesvirus saimiri, ECRF3, shows ligand-regulated signaling through Gi, Gq, and G 12/13 proteins but constitutive signaling only through Gi and G12/13 proteins," *Journal of Biological Chemistry*, vol. 279, no. 31, pp. 32524–32533, 2004.

[70] S. K. Ahuja and P. M. Murphy, "Molecular piracy of mammalian interleukin-8 receptor type B by herpesvirus saimiri," *Journal of Biological Chemistry*, vol. 268, no. 28, pp. 20691–20694, 1993.

[71] T. N. Kledal, M. M. Rosenkilde, and T. W. Schwartz, "Selective recognition of the membrane-bound CX3C chemokine, fractalkine, by the human cytomegalovirus-encoded broad-spectrum receptor US28," *FEBS Letters*, vol. 441, no. 2, pp. 209–214, 1998.

[72] P. Casarosa, Y. K. Gruijthuijsen, D. Michel et al., "Constitutive signaling of the human cytomegalovirus-encoded receptor UL33 differs from that of its rat cytomegalovirus homolog R33 by promiscuous activation of G proteins of the Gq, Gi, and Gs classes," *Journal of Biological Chemistry*, vol. 278, no. 50, pp. 50010–50023, 2003.

[73] P. Casarosa, R. A. Bakker, D. Verzijl et al., "Constitutive signaling of the human cytomegalovirus-encoded chemokine receptor US28," *Journal of Biological Chemistry*, vol. 276, no. 2, pp. 1133–1137, 2001.

[74] N. J. Davis-poynter, D. M. Lynch, H. Vally et al., "Identification and characterization of a G protein-coupled receptor homolog encoded by murine cytomegalovirus," *Journal of Virology*, vol. 71, no. 2, pp. 1521–1529, 1997.

[75] P. S. Beisser, C. Vink, J. G. van Dam, G. Grauls, S. J. V. Vanherle, and C. A. Bruggeman, "The R33 G protein-coupled receptor gene of rat cytomegalovirus plays an essential role in the pathogenesis of viral infection," *Journal of Virology*, vol. 72, no. 3, pp. 2352–2363, 1998.

[76] A. Alcami, "Viral mimicry of cytokines, chemokines and their receptors," *Nature Reviews Immunology*, vol. 3, no. 1, pp. 36–50, 2003.

[77] A. Alcami, "Interaction of viral chemokine inhibitors with chemokines," *Methods in Molecular Biology*, vol. 239, pp. 167–180, 2004.

[78] P. Najarro, H. J. Lee, J. Fox, J. Pease, and G. L. Smith, "Yaba-like disease virus protein 7L is a cell-surface receptor for chemokine CCL1," *Journal of General Virology*, vol. 84, pp. 3325–3336, 2003.

[79] P. Najarro, C. Gubser, M. Hollinshead, J. Fox, J. Pease, and G. L. Smith, "Yaba-like disease virus chemokine receptor 7L, a CCR8 orthologue," *Journal of General Virology*, vol. 87, pp. 809–816, 2006.

[80] S. J. Paulsen, M. M. Rosenkilde, J. Eugen-Olsen, and T. N. Kledal, "Epstein-barr virus-encoded BILF1 is a constitutively active G protein-coupled receptor," *Journal of Virology*, vol. 79, no. 1, pp. 536–546, 2005.

[81] P. S. Baisser, D. Verzijl, Y. K. Gruijthuijsen et al., "The Epstein-Barr virus BILF1 gene encodes a G protein-coupled receptor that inhibits phosphorylation of RNA-dependent protein kinase," *Journal of Virology*, vol. 79, no. 1, pp. 441–449, 2005.

[82] M. M. Rosenkilde, T. Benned-Jensen, H. Andersen et al., "Molecular pharmacological phenotyping of EBI2: an orphan seven-transmembrane receptor with constitutive activity," *Journal of Biological Chemistry*, vol. 281, no. 19, pp. 13199–13208, 2006.

[83] U. Gether, F. Asmar, A. K. Meinild, and S. G. F. Rasmussen, "Structural basis for activation of G-protein-coupled receptors," *Pharmacology and Toxicology*, vol. 91, no. 6, pp. 304–312, 2002.

[84] S. G. F. Rasmussen, A. D. Jensen, G. Liapakis, P. Ghanouni, J. A. Javitch, and U. Gether, "Mutation of a highly conserved aspartic acid in the β2 adrenergic receptor: constitutive activation, structural instability, and conformational rearrangement of transmembrane segment 6," *Molecular Pharmacology*, vol. 56, no. 1, pp. 175–184, 1999.

[85] W. M. Oldham and H. E. Hamm, "Heterotrimeric G protein activation by G-protein-coupled receptors," *Nature Reviews Molecular Cell Biology*, vol. 9, no. 1, pp. 60–71, 2008.

[86] B. Lagane, S. Ballet, T. Planchenault et al., "Mutation of the DRY motif reveals different structural requirements for the CC chemokine receptor 5-mediated signaling and receptor endocytosis," *Molecular Pharmacology*, vol. 67, no. 6, pp. 1966–1976, 2005.

[87] F. Hüttenrauch, A. Nitzki, F. T. Lin, S. Höning, and M. Oppermann, "β-arrestin binding to CC chemokine receptor 5 requires multiple C-terminal receptor phosphorylation sites and involves a conserved Asp-Arg-Tyr sequence motif," *Journal of Biological Chemistry*, vol. 277, no. 34, pp. 30769–30777, 2002.

[88] E. H. Schneider, D. Schnell, A. Strasser, S. Dove, and R. Seifert, "Impact of the DRY motif and the missing "ionic lock" on constitutive activity and G-protein coupling of the human histamine H4 receptor," *Journal of Pharmacology and Experimental Therapeutics*, vol. 333, no. 2, pp. 382–392, 2010.

[89] K. Takahashi, S. Tokita, and H. Kotani, "Generation and characterization of highly constitutive active histamine H3 receptors," *Journal of Pharmacology and Experimental Therapeutics*, vol. 307, no. 1, pp. 213–218, 2003.

[90] A. Rouleau, X. Ligneau, J. Tardivel-Lacombe et al., "Histamine H3-receptor-mediated [35S]GTPγ[S] binding: evidence for constitutive activity of the recombinant and native rat and human H3 receptors," *British Journal of Pharmacology*, vol. 135, no. 2, pp. 383–392, 2002.

[91] K. Kristiansen, "Molecular mechanisms of ligand binding, signaling, and regulation within the superfamily of G-protein-coupled receptors: molecular modeling and mutagenesis approaches to receptor structure and function," *Pharmacology and Therapeutics*, vol. 103, no. 1, pp. 21–80, 2004.

[92] A. E. Alewijnse, H. Timmerman, E. H. Jacobs et al., "The effect of mutations in the DRY motif on the constitutive activity and structural instability of the histamine H2 receptor," *Molecular Pharmacology*, vol. 57, no. 5, pp. 890–898, 2000.

[93] S. Okinaga, D. Slattery, A. Humbles et al., "C5L2, a nonsignaling C5A binding protein," *Biochemistry*, vol. 42, no. 31, pp. 9406–9415, 2003.

[94] S. C. Peiper, Z. X. Wang, K. Neote et al., "The duffy antigen/receptor for chemokines (DARC) is expressed in endothelial cells of duffy negative individuals who lack the erythrocyte receptor," *Journal of Experimental Medicine*, vol. 181, no. 4, pp. 1311–1317, 1995.

[95] M. Pruenster and A. Rot, "Throwing light on DARC," *Biochemical Society Transactions*, vol. 34, pp. 1005–1008, 2006.

[96] R. J. B. Nibbs, S. M. Wylie, J. Yang, N. R. Landau, and G. J. Graham, "Cloning and characterization of a novel

promiscuous human β-chemokine receptor D6," *Journal of Biological Chemistry*, vol. 272, no. 51, pp. 32078–32083, 1997.

[97] M. Weber, E. Blair, C. V. Simpson et al., "The chemokine receptor D6 constitutively traffics to and from the cell surface to internalize and degrade chemokines," *Molecular Biology of the Cell*, vol. 15, no. 5, pp. 2492–2508, 2004.

[98] P. J. Holst, H. R. Lüttichau, T. W. Schwartz, and M. M. Rosenkilde, "Virally encoded chemokines and chemokine receptors in the role of viral infections," *Contributions to Microbiology*, vol. 10, pp. 232–252, 2003.

[99] C. A. Flanagan, "A GPCR that is not 'DRY'," *Molecular Pharmacology*, vol. 68, no. 1, pp. 1–3, 2005.

[100] L. Arvanitakis, E. Geras-Raaka, A. Varma, M. C. Gershengorn, and E. Cesarman, "Human herpesvirus KSHV encodes a constitutively active G-protein- coupled receptor linked to cell proliferation," *Nature*, vol. 385, no. 6614, pp. 347–350, 1997.

[101] M. Burger, J. A. Burger, R. C. Hoch, Z. Oades, H. Takamori, and I. U. Schraufstatter, "Point mutation causing constitutive signaling of CXCR2 leads to transforming activity similar to Kaposi's sarcoma herpesvirus-G protein- coupled receptor," *Journal of Immunology*, vol. 163, no. 4, pp. 2017–2022, 1999.

[102] M. M. Rosenkilde, T. N. Kledal, P. J. Holst, and T. W. Schwartz, "Selective elimination of high constitutive activity or chemokine binding in the human herpesvirus 8 encoded seven transmembrane oncogene ORF74," *Journal of Biological Chemistry*, vol. 275, no. 34, pp. 26309–26315, 2000.

[103] M. N. Wakeling, D. J. Roy, A. A. Nash, and J. P. Stewart, "Characterization of the murine gammaherpesvirus 68 ORF74 product: a novel oncogenic G protein-coupled receptor," *Journal of General Virology*, vol. 82, pp. 1187–1197, 2001.

[104] D. Verzijl, C. P. Fitzsimons, M. Van Dijk et al., "Differential activation of murine herpesvirus 68- and Kaposi's sarcoma-associated herpesvirus-encoded ORF74 G protein-coupled receptors by human and murine chemokines," *Journal of Virology*, vol. 78, no. 7, pp. 3343–3351, 2004.

[105] Y. Isegawa, Z. Ping, K. Nakano, N. Sugimoto, and K. Yamanishi, "Human herpesvirus 6 open reading frame U12 encodes a functional β- chemokine receptor," *Journal of Virology*, vol. 72, no. 7, pp. 6104–6112, 1998.

[106] C. P. Fitzsimons, U. A. Gompels, D. Verzijl et al., "Chemokine-directed trafficking of receptor stimulus to different G proteins: selective inducible and constitutive signaling by human herpesvirus 6-encoded chemokine receptor U51," *Molecular Pharmacology*, vol. 69, no. 3, pp. 888–898, 2006.

[107] R. Case, E. Sharp, T. Benned-Jensen, M. M. Rosenkilde, N. Davis-Poynter, and H. E. Farrell, "Functional analysis of the murine cytomegalovirus chemokine receptor homologue M33: ablation of constitutive signaling is associated with an attenuated phenotype in vivo," *Journal of Virology*, vol. 82, no. 4, pp. 1884–1898, 2008.

[108] Y. K. Gruijthuijsen, E. V. H. Beuken, M. J. Smit, R. Leurs, C. A. Bruggeman, and C. Vink, "Mutational analysis of the R33-encoded G protein-coupled receptor of rat cytomegalovirus: identification of amino acid residues critical for cellular localization and ligand-independent signalling," *Journal of General Virology*, vol. 85, pp. 897–909, 2004.

[109] S. M. Rodems and D. H. Spector, "Extracellular signal-regulated kinase activity is sustained early during human cytomegalovirus infection," *Journal of Virology*, vol. 72, no. 11, pp. 9173–9180, 1998.

[110] J. Vomaske, R. M. Melnychuk, P. P. Smith et al., "Differential ligand binding to a human cytomegalovirus chemokine receptor determines cell type-specific motility," *PLoS Pathogens*, vol. 5, no. 2, Article ID e1000304, 2009.

[111] O. Pleskoff, P. Casarosa, L. Verneuil et al., "The human cytomegalovirus-encoded chemokine receptor US28 induces caspase-dependent apoptosis," *FEBS Journal*, vol. 272, no. 16, pp. 4163–4177, 2005.

[112] D. Maussang, D. Verzijl, M. Van Walsum et al., "Human cytomegalovirus-encoded chemokine receptor US28 promotes tumorigenesis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 35, pp. 13068–13073, 2006.

[113] D. Maussang, E. Langemeijer, C. P. Fitzsimons et al., "The human cytomegalovirus-encoded chemokine receptor US28 promotes angiogenesis and tumor formation via cyclooxygenase-2," *Cancer Research*, vol. 69, no. 7, pp. 2861–2869, 2009.

[114] E. Slinger, D. Maussang, A. Schreiber et al., "HCMV-encoded chemokine receptor US28 mediates proliferative signaling through the IL-6-STAT3 axis," *Science Signaling*, vol. 3, no. 133, Article ID ra58, 2010.

[115] P. Ranganathan, P. A. Clark, J. S. Kuo, M. S. Salamat, and R. F. Kalejta, "Significant association of multiple human cytomegalovirus genomic loci with glioblastoma multiforme samples," *Journal of Virology*, vol. 86, no. 2, pp. 854–864, 2012.

[116] K. G. Lucas, L. Bao, R. Bruggeman, K. Dunham, and C. Specht, "The detection of CMV pp65 and IE1 in glioblastoma multiforme," *Journal of Neuro-Oncology*, vol. 103, no. 2, pp. 231–238, 2011.

[117] C. S. Cobbs, "Evolving evidence implicates cytomegalovirus as a promoter of malignant glioma pathogenesis," *Herpesviridae*, vol. 2, no. 1, p. 10, 2011.

[118] K. Dziurzynski, J. Wei, W. Qiao et al., "Glioma-associated cytomegalovirus mediates subversion of the monocyte lineage to a tumor propagating phenotype," *Clinical Cancer Research*, vol. 17, no. 14, pp. 4642–4649, 2011.

[119] R. Lyngaa, K. Nørregaard, M. Kristensen, V. Kubale, M. M. Rosenkilde, and T. N. Kledal, "Cell transformation mediated by the Epstein-Barr virus G protein-coupled receptor BILF1 is dependent on constitutive signaling," *Oncogene*, vol. 29, no. 31, pp. 4388–4398, 2010.

[120] A. J. Butcher, R. Prihandoko, K. C. Kong et al., "Differential G-protein-coupled receptor phosphorylation provides evidence for a signaling bar code," *Journal of Biological Chemistry*, vol. 286, no. 13, pp. 11506–11518, 2011.

[121] A. J. Butcher, K. C. Kong, R. Prihandoko, and A. B. Tobin, "Physiological role of g-protein coupled receptor phosphorylation," *Handbook of Experimental Pharmacology*, vol. 208, pp. 79–94, 2012.

[122] S. K. Shenoy and R. J. Lefkowitz, "Multifaceted roles of β-arrestins in the regulation of seven-membrane-spanning receptor trafficking and signalling," *Biochemical Journal*, vol. 375, pp. 503–515, 2003.

[123] S. K. Shenoy, P. H. McDonald, T. A. Kohout, and R. J. Lefkowitz, "Regulation of receptor fate by ubiquitination of activated β2-adrenergic receptor and β-arrestin," *Science*, vol. 294, no. 5545, pp. 1307–1313, 2001.

[124] A. Fraile-Ramos, A. Pelchen-Matthews, T. N. Kledal, H. Browne, T. W. Schwartz, and M. Marsh, "Localization of HCMV UL33 and US27 in endocytic compartments and viral membranes," *Traffic*, vol. 3, no. 3, pp. 218–232, 2002.

[125] E. A. Berger, "HIV entry and tropism: the chemokine receptor connection," *AIDS*, vol. 11, pp. S3–16, 1997.

[126] A. Fraile-Ramos, T. N. Kledal, A. Pelchen-Matthews, K. Bowers, T. W. Schwartz, and M. Marsh, "The human cytomegalovirus US28 protein is located in endocytic vesicles and undergoes constitutive endocytosis and recycling," *Molecular Biology of the Cell*, vol. 12, no. 6, pp. 1737–1749, 2001.

[127] A. Fraile-Ramos, T. A. Kohout, M. Waldhoer, and M. Marsh, "Endocytosis of the viral chemokine receptor US28 does not require beta-arrestins but is dependent on the clathrin-mediated pathway," *Traffic*, vol. 4, no. 4, pp. 243–253, 2003.

[128] G. Alkhatib, C. Combadiere, C. C. Broder et al., "CC CKR5: a RANTES, MIP-1α, MIP-1β receptor as a fusion cofactor for macrophage-tropic HIV-1," *Science*, vol. 272, no. 5270, pp. 1955–1958, 1996.

[129] Y. Feng, C. C. Broder, P. E. Kennedy, and E. A. Berger, "HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor," *Science*, vol. 272, no. 5263, pp. 872–877, 1996.

[130] C.-Z. Dong, S. Tian, N. Madani et al., "Role of CXCR4 internalization in the anti-HIV activity of stromal cell-derived factor-1α probed by a novel synthetically and modularly modified-chemokine analog," *Experimental Biology and Medicine*, vol. 236, no. 12, pp. 1413–1419, 2011.

[131] N. Signoret, J. Oldridge, A. Pelchen-Matthews et al., "Phorbol esters and SDF-1 induce rapid endocytosis and down modulation of the chemokine receptor CXCR4," *Journal of Cell Biology*, vol. 139, no. 3, pp. 651–664, 1997.

[132] N. Signoret, M. M. Rosenkilde, P. J. Klasse et al., "Differential regulation of CXCR4 and CCR5 endocytosis," *Journal of Cell Science*, vol. 111, pp. 2819–2830, 1998.

[133] P. J. Klasse, M. M. Rosenkilde, N. Signoret, A. Pelchen-Matthews, T. W. Schwartz, and M. Marsh, "CD4-chemokine receptor hybrids in human immunodeficiency virus type 1 infection," *Journal of Virology*, vol. 73, no. 9, pp. 7453–7466, 1999.

[134] M. Oppermann, M. Mack, A. E. I. Proudfoot, and H. Olbrich, "Differential effects of CC chemokines on CC chemokine receptor 5 (CCR5) phosphorylation and identification of phosphorylation sites on the CCR5 carboxyl terminus," *Journal of Biological Chemistry*, vol. 274, no. 13, pp. 8875–8885, 1999.

[135] I. Aramori, J. Zhang, S. S. G. Ferguson, P. D. Bieniasz, B. R. Cullen, and M. G. Caron, "Molecular mechanism of desensitization of the chemokine receptor CCR-5: receptor signaling and internalization are dissociable from its role as an HIV-1 co-receptor," *The EMBO Journal*, vol. 16, no. 15, pp. 4606–4616, 1997.

[136] K. Kraft, H. Olbrich, I. Majoul, M. Mack, A. Proudfoot, and M. Oppermann, "Characterization of sequence determinants within the carboxyl-terminal domain of chemokine receptor CCR5 that regulate signaling and receptor internalization," *Journal of Biological Chemistry*, vol. 276, no. 37, pp. 34408–34418, 2001.

[137] S. Venkatesan, A. Petrovic, M. Locati, Y. O. Kim, D. Weissman, and P. M. Murphy, "A membrane-proximal basic domain and cysteine cluster in the C-terminal tail of CCR5 constitute a bipartite motif critical for cell surface expression," *Journal of Biological Chemistry*, vol. 276, no. 43, pp. 40133–40145, 2001.

[138] M. J. Orsini, J. L. Parent, S. J. Mundell, and J. L. Benovic, "Trafficking of the HIV coreceptor CXCR4. Role of arrestins and identification of residues in the C-terminal tail that mediate receptor internalization," *Journal of Biological Chemistry*, vol. 274, no. 43, pp. 31076–31086, 1999.

[139] B. Haribabu, R. M. Richardson, I. Fisher et al., "Regulation of human chemokine receptors CXCR4: role of phosphorylation in desensitization and internalization," *Journal of Biological Chemistry*, vol. 272, no. 45, pp. 28726–28731, 1997.

[140] T. Kawai, U. Choi, N. L. Whiting-Theobald et al., "Enhanced function with decreased internalization of carboxy-terminus truncated CXCR4 responsible for WHIM syndrome," *Experimental Hematology*, vol. 33, no. 4, pp. 460–468, 2005.

[141] M. P. Stropes, O. D. Schneider, W. A. Zagorski, J. L. C. Miller, and W. E. Miller, "The carboxy-terminal tail of human cytomegalovirus (HCMV) US28 regulates both chemokine-independent and chemokine-dependent signaling in HCMV-infected cells," *Journal of Virology*, vol. 83, no. 19, pp. 10016–10027, 2009.

[142] B. Bodaghi, T. R. Jones, D. Zipeto et al., "Chemokine sequestration by viral chemoreceptors as a novel viral escape strategy: withdrawal of chemokines from the environment of cytomegalovirus- infected cells," *Journal of Experimental Medicine*, vol. 188, no. 5, pp. 855–866, 1998.

[143] T. Mokros, A. Rehm, J. Droese, M. Oppermann, M. Lipp, and U. E. Höpken, "Surface expression and endocytosis of the human cytomegalovirus-encoded chemokine receptor US28 is regulated by Agonist-independent phosphorylation," *Journal of Biological Chemistry*, vol. 277, no. 47, pp. 45122–45128, 2002.

[144] M. Waldhoer, P. Casarosa, M. M. Rosenkilde et al., "The carboxyl terminus of human cytomegalovirus-encoded 7 transmembrane receptor US28 camouflages agonism by mediating constitutive endocytosis," *Journal of Biological Chemistry*, vol. 278, no. 21, pp. 19473–19482, 2003.

[145] L. K. Stapleton, K. L. Arnolds, A. P. Lares, T. M. Devito, and J. V. Spencer, "Receptor chimeras demonstrate that the C-terminal domain of the human cytomegalovirus US27 gene product is necessary and sufficient for intracellular receptor localization," *Virology Journal*, vol. 9, no. 1, article 42, 2012.

[146] C. Liu, G. Sandford, G. Fei, and J. Nicholas, "Galpha protein selectivity determinant specified by a viral chemokine receptor-conserved region in the C tail of the human herpesvirus 8 g protein-coupled receptor," *Journal of Virology*, vol. 78, no. 5, pp. 2460–2471, 2004.

[147] M. Schwarz and P. M. Murphy, "Kaposi's sarcoma-associated herpesvirus G protein-coupled receptor constitutively activates NF-κB and induces proinflammatory cytokine and chemokine production via a C-terminal signaling determinant," *Journal of Immunology*, vol. 167, no. 1, pp. 505–513, 2001.

[148] D. Verzijl, L. Pardo, M. Van Dijk et al., "Helix 8 of the viral chemokine receptor ORF74 directs chemokine binding," *Journal of Biological Chemistry*, vol. 281, no. 46, pp. 35327–35335, 2006.

[149] J. Zuo, L. L. Quinn, J. Tamblyn et al., "The Epstein-Barr virus-encoded BILF1 protein modulates immune recognition of endogenously processed antigen by targeting major histocompatibility complex class I molecules trafficking on both the exocytic and endocytic pathways," *Journal of Virology*, vol. 85, no. 4, pp. 1604–1614, 2011.

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Review Article

Orthopoxvirus Genes That Mediate Disease Virulence and Host Tropism

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In the course of evolution, viruses have developed various molecular mechanisms to evade the defense reactions of the host organism. When understanding the mechanisms used by viruses to overcome manifold defense systems of the animal organism, represented by molecular factors and cells of the immune system, we would not only comprehend better but also discover new patterns of organization and function of these most important reactions directed against infectious agents. Here, study of the orthopoxviruses pathogenic for humans, such as variola (smallpox), monkeypox, cowpox, and vaccinia viruses, may be most important. Analysis of the experimental data, presented in this paper, allows to infer that variola virus and other orthopoxviruses possess an unexampled set of genes whose protein products efficiently modulate the manifold defense mechanisms of the host organisms compared with the viruses from other families.

1. Introduction

In the course of evolution, viruses have developed various molecular mechanisms allowing them to evade the host's defense reactions [1–3]. Viruses can become particularly dangerous when they evolve to acquire the possibility to infect new animal species [4, 5]. The defense systems of the new host may be generally unable to counteract the new pathogen and many individuals will die. In any epidemics, there are also individuals showing little sensitivity or complete resistance to the particular pathogen. Both increased sensitivity and resistance to the infection are specified by the individual's genetic makeup and various environmental factors. Accordingly, mass epidemics not only produce new virus variants but also alter the host population structure: highly sensitive individuals die, while the portion of resistant individuals in the population increases. Therefore, the coevolution of the virus and the host is a mutually dependent process. It should be noted that mutational frequencies that drive genetic variations in viruses are much higher than in mammals [6]. On the other hand, animal genomes contain incomparably higher numbers of genes, while virus resistance mutations usually affect one or, less frequently, several genes. Such mutations may affect virus adsorption on the target cells, its replication, and/or the evasion of the host's defense systems.

Poxviruses are the largest mammalian DNA viruses with the developmental cycle taking place in the cellular cytoplasm [7]. These viruses encode a large set of proteins providing for extranuclear synthesis of virus mRNAs, replication of virus DNA, and assembly of complex virions and are involved in the regulation of multifactorial interactions of the virus with both individual cells and infected host organism. The unique properties of poxviruses attract close attention of researchers. The viruses belonging to the genus *Orthopoxvirus* are best studied among other viruses of the family Poxviridae, because this genus includes four virus species pathogenic for humans: variola (smallpox) virus (VARV), monkeypox virus (MPXV), cowpox virus (CPXV), and vaccinia virus (VACV). These orthopoxviruses are immunologically cross-reactive and cross-protective, so that infection with any member of this genus provides

protection against an infection with any other member [3]. An important experimental model is ectromelia virus (mousepox virus, ECTV) [8, 9].

VARV causes smallpox and is an exclusively anthroponotic agent. For years, this human pathogen caused epidemics of disease with mortalities of 10–40%. Only the coordinated efforts of the world community, under the aegis of the World Health Organization, accomplished the eradication of smallpox [3, 10].

Natural reservoir of MPXV is rodents. Human monkeypox resembles the clinical course of smallpox that was prevalent on the African continent and is recorded predominantly in Central and Western Africa [11, 12]. Its mortality rate in several studied human monkeypox outbreaks in Central Africa reached 16% [3, 11]. The specific feature of human monkeypox clinical course, distinguishing it from smallpox, is lymphadenitides. Another difference between the human monkeypox and smallpox is in that the human-to-human transmission efficiency of MPXV is considerably lower as compared with VARV [3]. That is why this virus has not so far caused any expanded epidemics.

CPXV displays the widest host range among the orthopoxviruses. Generally, human cowpox is a benign disease manifesting itself by isolated local lesions [3]. Human cowpox is recorded in the majority of European countries. Rodents (the main natural reservoir) or home pets and cattle (bridging hosts) represent the main sources of human CPXV infection [13–15]. In immunocompromised persons cowpox virus can cause a generalized eruption [16, 17] with lethal outcome in some cases [18].

VACV, used for vaccinating humans against smallpox, can be transmitted to man accidentally by contact with a vaccinee. Last years the number of reported outbreaks of the human diseases caused by the zoonotic VACV-like viruses is increasing in several countries [19–21].

VARV infection is a rare example of a strict anthroponosis caused by a virus propagating and spreading only within human populations; it is highly pathogenic for humans, being well adapted to overcome the defense barriers of this particular host. MPXV, CPXV, and VACV are zoonotic viruses with a wide range of sensitive species; they are evolutionary adapted to propagate in different mammalian hosts. In humans, they cause relatively rare sporadic disease cases when the virus is transmitted from an affected animal to a human [3]. ECTV, similarly to VARV, has a very narrow host range, being highly pathogenic only for certain mouse strains [8].

It is believed that viruses during coevolution with the host organism had incorporated into their genomes the coding sequences of various cellular genes and modified them for adapting to provide for their viability and preservation in the biosphere [22, 23]. Acquiring the knowledge about how viruses overcome numerous protective systems of mammals, which are represented by molecular factors and cells of the immune system, we will not only get a deeper understanding but also discover new patterns in organization and functioning of these most important mammalian organism responses directed against infectious agents.

2. Suppression of Molecular Recognition of Viruses by Innate Immune Cells

Innate immune cells express a large repertoire of germ line-encoded pattern recognition receptors (PRRs) that recognize microbial components. The receptors include toll-like receptors (TLRs), nod-like receptors (NLRs), RIG-1-like receptors (RLRs), and AIM2-like receptors (ALRs) [24]. These PRRs bind microbial ligands and initiate signaling cascades which result in the activation of transcription factors such as nuclear factor kappa B (NF-κB), interferon regulatory factors (IRFs), and activating protein-1 (AP-1) involved in the expression of inflammatory and type I interferon (IFN) genes [1].

In response to infection, cells constituting the mammalian innate immune system, such as macrophages and dendritic cells, produce proinflammatory cytokines. IL-1β and IL-18 are synthesized as cytoplasmic precursors, which should be cleaved by a cysteine protease termed caspase-1 to acquire the active form. Caspase-1, in turn, is also synthesized as an inactive precursor, which can be activated within a large cytosolic protein complex called inflammasome [25–27]. Inflammasomes act as intracellular sensors responsive to conserved microbial components similarly to the functioning of TLRs on the cell surface or in endosomes. Proteins of the TLR family possess an intracellular TIR domain, which responds to infection by triggering intracellular signal cascades that activate innate immune reactions.

It was revealed that orthopoxvirus genomes contain two genes of TIR-containing proteins: VACV A46 and A52 [28–30]. These proteins have different functions and specifically inhibit intracellular signal cascades activating transcription factor NF-κB, critical for innate immunity. A46 interacts with the factors MyD88, TIRAP, TRAM, and TRIF, and A52 with IRAK2 and TRAF6. It should be noted that CPXV and some VACV strains encode both above proteins, while VARV and MPXV, which are most pathogenic for humans, do not produce A52 VACV isologs (Table 1).

The crystallographic structure of A52 has been solved showing that this one is homodimer with folding similarity to B-cell-lymphoma- (Bcl-) 2-like proteins whose members inhibit apoptosis or activation of proinflammatory transcription factors [31]. To date a set of Bcl-2-like orthopoxviral proteins was discovered and characterized [32, 33] (Table 1). While these proteins share structural similarity, their degree of amino acid similarity is low indicating that they diverged long ago and although they share an ability to manipulate innate immune signaling pathways, they differ in their targets (Table 1) and mechanisms of action [32].

The crystallographic structure of VACV protein N1 (see Table 1) identified a groove similar to those of cellular antiapoptotic Bcl-2 proteins. N1 is therefore unusual in its dual ability to modulate both apoptosis and inflammatory signaling. N1 inhibits proapoptotic and proinflammatory signaling using independent surfaces of the protein [34]. Analyses of the other available three-dimensional structures of the orthopoxviral Bcl-2-like proteins have shown that

Table 1: Orthopoxviral Bcl-2-like proteins.

Protein function	VACV-COP		CPXV-GRI		MPXV-ZAI		VARV-IND	
	ORF	Size, aa	ORF	Size, aa	ORF	Size, aa	ORF	Size, aa
Inhibition of NF-κB and IRF3 activation by interacting with MyD88, TIRAP and TRIF, and TRAM	A46R	214	A49R	240	A47R	240	A52R	240
Inhibition of NF-κB activation by interacting with IRAK2 and TRAF6	A52R	190	A55R	190	—	—	J6R	71
Inhibition of NF-κB activation by interacting with IKK complex	B15R	149	B13R	149	B13R	149	B14R	149
Inhibition of NF-κB and IRF3 activation by interacting with IKK complex and TBK1, apoptosis inhibitor	N1L	117	Q1L	117	P1L	117	P1L	117
Inhibition of NF-κB and IRF3 activation by interacting with IRAK2, TRAF6, and DDX3	K7R	149	M6R	149	C6R	149	C4R	149
Inhibition of IRF3 and IRF7 activation by interacting with TANK, NAP1, and SINTBAD	C6L	151	C14L	156	D11L	153	D9L	156
Unknown	C1L	224	C19L	231	D19L	214	D14L	214
Unknown	C16L	181	D5L	153	—	—	D1L	153
Unknown	N2L	175	Q2L	175	P2L	177	P2L	177

VARV-IND: VARV strain India,1967, MPXV-ZAI: MPXV strain Zaire-I-96, CPXV-GRI: CPXV strain GRI-90, VAC-COP: VACV strain Copenhagen. ORF: open reading frame; aa: protein size in number of amino acid residues.

VACV proteins A52, B15, and K7 do not contain BH3-peptide-binding groove important for inhibition of apoptotic stimuli and these proteins inhibit only activation of proinflammatory transcription factors [31, 32].

Based on the sequence/structure similarity, it was proposed that additional VACV proteins N2, C1, and C16/B22 (and their orthopoxviral isologs) might have a similar role in suppression of PRR-induced host immune response as other studied Bcl-2-like proteins (Table 1), by antagonizing at different levels with the TLR signaling pathways [32, 34].

Thus, orthopoxviruses have a multigenic system controlling their recognition by innate immune cells. The detected distinctions between the Bcl-2-like genes of VARV, MPXV, CPXV, and VACV require further studies of the properties of the corresponding proteins.

3. The Ubiquitin-Proteasome Pathway in Viral Infection

It has been recently discovered that protein degradation is an especially important regulatory cell process [35, 36]. In the majority of cases, proteins in eukaryotic cells are degraded via an ubiquitin-directed pathway. Ubiquitin (Ub) comprises 76 amino acid (aa) residues and is among the most evolutionarily conserved polypeptides; Ub is covalently attached to target proteins by a coordinated action of three enzyme classes [37].

The ubiquitin-activating enzyme (E1) cleaves ATP to form a thioester bond between the Ub C-end and the cysteine in the active site of this enzyme. Thus, activated Ub is then transferred to the ubiquitin-conjugating enzyme (E2), also forming a thioester bond. E2–Ub interacts with ubiquitin-protein ligase (E3), which concurrently binds the target protein, frequently named the substrate. E3 causes transfer of the Ub from E2–Ub complex to the substrate by formation of the covalent isopeptide bond between the Ub C-end and the lysine residues in target protein (substrate). Attachment of a single Ub can change the function or localization of the protein in the cell. A tandem attachment of Ub molecules, producing a polyubiquitin chain, can also modify the function or cell localization of target protein or causes involvement of such protein in degradation by the cellular 26S proteasome leading to protein cleavage into short peptides and Ub release [35].

Ubiquitin is the first member of the ever increasing family of ubiquitin-like (Ubl) proteins, which are also involved in modification of various proteins and their functions. Such modification processes are frequently of transient character because of existence of Ub/Ubl-deconjugating enzymes (Ub/Ubl-specific proteases) along with Ub/Ubl-conjugating enzymes. It has been discovered that Ubl attachment to target protein can frequently enhance the interaction of this modified protein with other proteins or, on the contrary, block its interaction with the target [37].

The most numerous group of E3 ligases contains cullin-RING ubiquitin ligases (CRLs), multisubunit complexes comprising cullin proteins [38], RING H2 finger proteins (designated Rbx1, Roc1, or Hrt1) [39], variable substrate-recognition subunit (SRS), and, for the majority of CRLs, additional adaptor proteins uniting SRS with other CRL proteins [36].

Proteins of the cullin family are hydrophobic proteins playing the role of a backbone for assembly of the CRL complex [36, 38]. The CRL containing cullin-1 (CUL1), named SCF complex, has been most intensively studied. This complex comprises four subunits—Skp1, CUL1, F-box-containing protein, and Rbx1 (Figure 1(a)). The N-end of CUL1 protein binds to the Skp1 adaptor, which, in turn, interacts with F-box-containing protein. The C-terminal part

of CUL1 binds to Rbx1 protein, whose function is in the interaction with E2-Ub. In turn, F-box-containing protein [40, 41] provides for the interaction with substrate protein, which is ubiquitinated by the complex (see Figure 1(a)).

The CUL3-containing CRL complexes contain Rbx1; however, they differ from the other studied CRL classes by the absence of adaptor proteins [42]. The BTB-domain-containing protein, accomplishing interaction with substrate protein of the complex by another protein-protein binding domain [43], directly interacts with the N-terminal CUL3 region (see Figure 1(b)).

Thus, the information accumulated so far demonstrates that a tremendous diversity of CRL complexes can be formed in mammalian cells. This agrees with the modern understanding that the modification of proteins by ubiquitin or ubiquitin-like polypeptides is important for the fate and functioning of the majority of proteins in eukaryotic cell and can be involved in regulation of various biological processes [44–46].

Taking into account the importance of ubiquitin-ligase and ubiquitin-proteasome systems for the function of eukaryotic cells, the role of viruses in regulation of these processes has been intensively studied recently. Although the data on this topic are sparse, it has been already discovered that viruses of various families can influence the protein ubiquitination to overcome the cell defense mechanisms, including apoptosis, type I interferon response, and antigen presentation by the class I major histocompatibility complex [45, 47, 48].

The developmental cycle of orthopoxviruses takes place in the cell cytoplasm. Orthopoxviruses replicate in the discrete cytoplasmic structures called virus factories or virosomes. These structures are encompassed by endoplasmic reticulum membranes, resembling cytoplasmic mininuclei [49].

Recent experiments with VACV have demonstrated that proteasome inhibitors interfere with formation of virus factories in the cytoplasm of permissive cells and, as a consequence, lead to a radical decrease in virus replication [50, 51]. These results suggest that a normal development of orthopoxvirus infection requires a functioning ubiquitin-proteasome system.

Since it has been discovered that ubiquitin constitutes at least 3% of the total protein in VACV virions [52], this suggests that either the ubiquitin-ligase system is important for modification of virus proteins and assembly of virus particles or ubiquitin-modified proteins are packaged into virions for further involvement in the early infection stages in sensitive cells.

Metabolism of animal viruses depending on their specific features is directed to utilize the structures of the cell cytoplasmic or nuclear protein skeleton. In particular, it is assumed that the virus-specific cytopathic effect is determined not by cell damage meaningless from the virus standpoint but rather by a specific rearrangement of cytoskeleton elements that would create the conditions for virus reproduction [53, 54]. The cytoskeleton proteins are encoded by a large set of various genes with a tissue-specific expression. This determines the difference between the compositions of protein "backbone" in different cell types, which influences the functions of these cells [55]. These differences can influence the parameter of virus replication in the host organism, such as tissue tropism. In addition, the protein composition of cytoskeleton in various mammalian species (or cell cultures) can influence the overall sensitivity to certain viruses and determine the so-called host range. It is known that individual orthopoxvirus species considerably differ in the range of animal species where they can reproduce [56].

3.1. Viral Ankyrin-F-Box Containing Proteins. It is known that viral infections activate the cell antiviral signaling and inflammatory responses. The nuclear factor NF-κB, which regulates transcription of the genes involved in development of the apoptosis, inflammation, immune response, and cell proliferation [57], plays an important role in these responses. Activation of NF-κB is controlled by ankyrin (ANK) repeat containing proteins of the IκB family, which interact with this factor. In an inactive form, NF-κB (the dimer p65/p50) is bound to the inhibitory protein IκBα which via six ANK repeats interacts with p65 subunit. In response to molecular signals of infection, IκBα kinase (IKK) is phosphorylated by cellular protein kinase to phosphorylate IκBα at the serine residues at positions 32 and 36. The phosphorylated IκBα is polyubiquinated by the SCF complex at lysine 48 and degraded by the 26S proteasome complex, thereby removing the NF-κB inhibition; this factor moves to the cell nucleus and stimulates gene transcription via the interaction with specific DNA sequences [57, 58].

Different VACV strains inhibit activation of the cellular transcription factor NF-κB, thereby providing inhibition of inflammatory response development, which is among the first reactions of nonspecific protection from infectious agents. It was demonstrated that the highly attenuated VACV strain MVA failed to inhibit NF-κB activation. Recombination-based introduction of the *K1L* gene from VACV strain WR to the MVA genome restored the ability of the virus to inhibit the activation of cellular factor NF-κB [59]. VACV protein K1 belongs to the family of ANK proteins [60–62] and it was assumed that K1 can inhibit degradation of the cellular IκBα via competing with it for phosphorylation by the enzyme IKK and subsequent ubiquitination and degradation. Another ANK-containing protein, CPXV C9 (not synthesized by VACV; Table 1) is likely to act in an analogous manner [58] and, consequently, is able to rescue the mutation VACV *K1L*$^-$ [63].

Our analysis has demonstrated [62–64] that orthopoxviruses code for a large set of ANK proteins (Table 2). This is the largest family of orthopoxvirus proteins; moreover, each species has its specific set of the corresponding genes [63]. Of the natural orthopoxviruses, CPXV, displaying the widest host range, has 14 unique ANK genes in its genome (two of them are duplicated in the terminal genomic regions); MPXV, eight such genes and VARV, a stringently anthroponotic virus, five ANK genes, VACV-COP encodes five ANK proteins (three match the VARV proteins), while the highly attenuated variant VACV-MVA, obtained by multiple passages on chorioallantoic membranes (CAMs) of

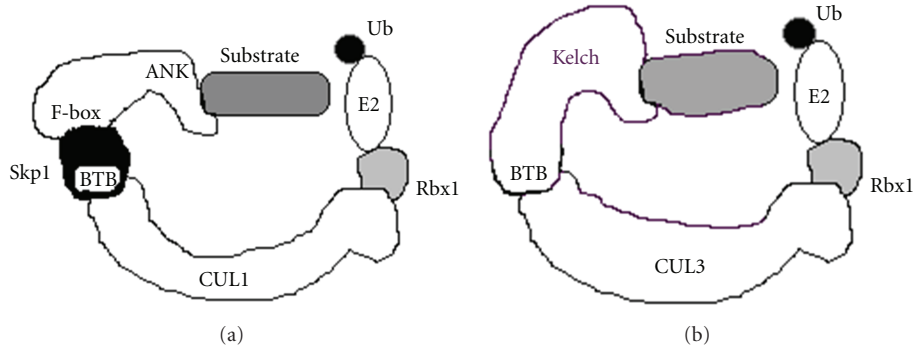

Figure 1: A schematic of two classes of orthopoxvirus E3 ubiquitin ligases: (a) SCF E3 ligase and (b) BTB-kelch/Cul3 E3 ligase.

chick embryos and having a very narrow range of sensitive cell cultures, retained only one ANK gene [65].

It has been shown that many poxvirus ANK proteins contain F-box sequences at their C-ends [66]. Such combination of domains is characteristic of poxviruses only. In the cellular proteins, F-box domain is usually localized to the N-terminal part. In addition, the combination of ANK and F-box domains has not been found in cellular proteins [67, 68]. Our analysis allowed to detect F-box sequences in the C-terminal regions in 13 of the 14 CPXV ANK proteins (Table 1) [63].

Recently, it has been experimentally demonstrated for the ANK-F-box proteins C9 (CHOhr) of CPXV [58], G1 of VARV (D3/H3 in CPXV) [69], 186R of VACV-MVA (B16 in CPXV) [70], as well as EVM002, EVM005, and EVM154 of ECTV (D3/H3, D8, and B3 in CPXV) [71] that they interact with the cellular SCF complex (Figure 1(a)) and, presumably, provide for specific interaction with substrate proteins of cellular or viral origins, which are then ubiquitinated by this complex. An important problem is to detect these substrate proteins for each of the numerous orthopoxvirus proteins of the considered family.

3.2. Viral BTB-Kelch-Like Proteins. Among all the viruses, only the representatives of the family Poxviridae contain the genes of kelch-like proteins in their genomes. According to structural similarity, they are ascribed to the same group as *Drosophila* kelch protein (BTB-kelch) [72–74]. These proteins contain the N-terminal BTB domain and C-terminal kelch domain. Computer analysis of orthopoxvirus genomes has demonstrated that CPXV codes for six BTB-kelch family proteins with a size of about 500 AA residues each and mutual identity in AA sequence in the range of 22–26%. VACV genome codes for only three full-sized kelch-like proteins, which are highly homologous to the corresponding CPXV proteins; as for the highly attenuated strain VACV-MVA, unable to replicate in the majority of mammalian cell lines, it retained only one gene of this family [65]. The same gene is the only gene in MPXV genome encoding a BTB-kelch protein. As for VARV genome, all genes of this family are destroyed due to multiple mutations; consequently, only short potential ORFs, which are nonfunctional fragments of the genes of a precursor virus, are detectable in this virus [74] (Table 3). ECTV codes for four genes of the considered family—*EVM18*, *EVM27*, *EVM150*, and *EVM167*, which correspond to the CPXV-GRI genes *C18L*, *G3L*, *A57R*, and *B19R* [23, 75].

The absence of the genes from this family in various VARV isolates and the possibility of their deleting in VACV without any loss in its viability in cell culture [76] indicate that these genes are not vitally important for orthopoxvirus replication in cultured cells. Presumably, these genes are important for manifestation of species-specific properties of orthopoxviruses *in vivo*. It has been assumed that these genes can play a role in adaptation, that is, they can determine the host range (tissue tropism) and/or the possibility of virus persistence in animal body [23]. In particular, CPXV, low pathogenic for humans and displaying the widest range of sensitive animals in nature, codes for the largest set of BTB-kelch proteins. In VARV, highly pathogenic for its only host, human, in organism of which this virus cannot persist, all the genes of BTB-kelch subfamily are mutationally destroyed [63, 64].

The available data suggest that various BTB-kelch proteins interact with CUL3 (Figure 1(b)) rather than with the other cullins, that is, BTB-kelch proteins are substrate-specific adaptors for CUL3 ubiquitin-ligase complex and regulate modification and/or degradation of various proteins [42].

When studying the properties of orthopoxvirus BTB-kelch proteins, it has been found that the ECTV proteins EVM150 and EVM167 are involved in formation of active CUL3-containing ubiquitin ligases [77]. Two other proteins, EVM18 and EVM27, also interact with CUL3 [78]. Since the mutual homology of these viral proteins is low, it is likely that their functions are different and they interact with different targets. It has been experimentally demonstrated that a directed deletion of individual genes encoding EVM18, EVM27, or EVM167 radically decreases the ECTV virulence for white mice, while the damage of EVM150 gene has no effect on the virulence [79].

Deletion of four CPXV *BTB-kelch* genes led to a decrease in the cytopathic effect on cell culture and statistically significant reduction in formation of the virus-induced cytoplasmic pseudopodia [80, 81].

Table 2: Orthopoxviral ankyrin-F-box-like proteins.

VACV-COP		CPXV-GRI		MPXV-ZAI		VARV-IND	
ORF	Size, aa	ORF	Size, aa	ORF	Size, aa	ORF	Size, aa
C19L*	259	***D3L****	**586**	**J3L***	**587**	None	None
C17L*	386	**D4L***	**672**	None	None	None	None
None	None	***D8L***	**661**	None	None	None	None
None	None	**D14L**	**764**	None	None	None	None
None	None	**C1L**	**437**	**D1L***	**437**	None	None
None	None	**C3L**	**833**	None	None	None	None
None	None	***C9L***	**668**	**D7L**	**660**	D6L	452
C9L	**634**	**C11L**	**614**	**D9L**	**630**	D7L	153
M1L	**472**	**O1L**	**474**	**O1L**	**442**	**O1L**	**446**
K1L	284	M1L	284	C1L	284	C1L	66
B4R	**558**	***B3R***	**558**	**B5R**	**561**	**B6R**	**558**
B18R	**574**	***B16R***	**574**	None	None	**B19R**	**574**
B20R	127	**B18R**	**795**	**B17R**	**793**	**B21R**	**787**
B21R*	91	**K1R**	**581**	None	None	None	None
None	None	None	None	**N4R***	**437**	None	None
B23R*	386	**H2R***	**672**	None	None	None	None
B25R*	259	***H3R****	**586**	**J1R***	**587**	**G1R**	**585**

Asterisks denote ORFs that are duplicated in left and right inverted terminal repeat regions of the viral genome. ORFs with full length are set in bold. The ORFs for the proteins with experimentally confirmed interaction with the cellular Cullin1-containing ubiquitin-protein ligase are indicated by bold italic letters.

Table 3: Orthopoxviral BTB-kelch-like proteins.

VACV-COP		CPXV-GRI		MPXV-ZAI		VARV-IND	
ORF	Size, aa	ORF	Size, aa	ORF	Size, aa	ORF	Size, aa
None	None	**D11L**	**521**	None	None	None	None
C2L	**512**	***C18L***	**512**	D18L	107	None	None
F3L	**480**	***G3L***	**485**	**C9L**	**487**	C7L	179
A55R	**564**	***A57R***	**564**	None	None	J7R	71
B10R	166	**B9R**	**501**	None	None	None	None
None	None	***B19R***	**557**	B18R	70	B22R	70

ORFs with full length are set in bold. The ORFs for the proteins with experimentally confirmed interaction with the cellular Cullin 3-containing ubiquitin-protein ligase are indicated by bold italic letters.

Deletion of individual *kelch*-like genes in the VACV genome provided for demonstrating that the damage of genes *C2L* or *A55R* (see Table 2) led to similar effects, appearing as changes in the morphology of virus plaques on cell culture monolayer, decrease in virus-induced cytoplasmic pseudopodia, decrease in Ca^{2+}-independent adhesion of VACV-infected cells, and induction of larger lesions in the model of intradermal infection of mouse ear pinnae as compared with the wildtype virus [82, 83]. Damage of the VACV *kelch*-like gene *F3L* did not cause so pronounced effects [84]. Interestingly, this particular single *BTB-kelch* gene remained in MPXV (see Table 3) and the highly attenuated VACV strain MVA.

The ability of orthopoxvirus BTB-kelch-like proteins to interact with Cullin-3-containing ubiquitin-protein ligase to a considerable degree relates this family to the family of orthopoxvirus ankyrin-F-box-like proteins interacting with Cullin-1-containing ubiquitin-protein ligase. Most likely, the proteins of these two families are involved in organization of the multifactorial intricate system of interactions of virus proteins with one another and cellular components. We believe that such interactions can determine a wide range of animal tissues and species sensitive to CPXV as well as for the tolerant mode of relationships between this virus and the host. One can speculate that destruction of the majority of the genes/proteins belonging to these two families, characteristic of VARV, is the most likely reason underlying a drastic narrowing of the VARV host range and its transition to an "aggressor" mode [63]. Note that VARV retained five genes encoding ankyrin-F-box proteins (Table 2), whereas the genes for BTB-kelch proteins are completely destroyed (Table 3).

4. Viral Apoptosis Inhibitors

One of the first lines of the organism's nonspecific defense against infectious agents and probably one of the most ancient ones is apoptosis (programmed cell death) [85, 86]. After a cell has been infected by a virus, apoptosis serves to kill the cell, thus preventing virus proliferation and protecting nearby cells from the infection. Apoptosis is a very common phenomenon in multicellular organisms. It is primarily mediated by cysteine proteases termed caspases. An important role in apoptosis regulation belongs to mitochondria and Bcl-2 proteins. Interferon-induced synthesis of RNase L (see Section 5) causes apoptosis mediated by caspases 8, 9, and 2 [87].

A key function in the induction of programmed cell death is performed by cellular caspase 1, which specifically cleaves inactive prointerleukin-1β and producing the mature interleukin-1β form (IL-1β). It should be noted that IL-1β itself is not associated with apoptosis; that is, it is other proteins that caspase-1 targets when triggering programmed cell death [88]. It was revealed that the product of the CPXV gene *SPI-2* is an inhibitor of caspases 1 and 8 and, therefore, an apoptosis inhibitor [89, 90]. A comparison of SPI-2 amino acid sequences showed that CPXV and VACV proteins are very similar but differ significantly from the VARV isolog [3].

Based on an *in silico* amino acid sequence analysis, another VACV protein, C12 (in VARV-IND, B25; see Table 4), was also classified into the same family of protease inhibitors as SPI-2 and named SPI-1; it was shown to act as an apoptosis inhibitor, too. However, the principle of its action so far remains unclear. Supposedly, SPI-1 inhibits a caspase-independent apoptosis pathway [91].

VACV protein F1 is localized in mitochondria and acts as a caspase 9 inhibitor, suppressing the programmed death of infected cells [92–94]. The amino acid sequence of another conserved orthopoxvirus protein, N1 (VACV-COP, Table 4), has little homology to the Bcl-2 sequence, but its tertiary structure closely resembles the proteins of this family, and N1 is an apoptosis inhibitor (see additionally Section 2) [34, 95].

Some VACV strains, as well as the camelpox virus, contain a gene of a transmembrane protein of 237 amino acids located in the Golgi apparatus, which suppresses apoptosis of infected cells [96]. CPXV encodes a somewhat shorter version of the same protein (T1), while VARV and MPXV lack gene of the isolog protein (Table 4).

Double-stranded RNA apparently also can induce apoptosis, as suggested by investigation of a VACV strain carrying a mutant *E3L* variant. It was shown that disruption of this gene results not only in increased interferon sensitivity of the virus (see Section 5) but also in activation of apoptosis of infected cells [97]. This gene is fairly well conserved among different orthopoxvirus species.

The first orthopoxvirus ubiquitin ligase belonging to the family of mono-subunit RING-containing E3 ligases was found in ECTV. First, it was shown that the RING domain-containing ECTV protein p28 is a virulence factor inhibiting TNF-induced apoptosis [98]. This viral protein localized in cytoplasmic virus factories is essential for virus replication in macrophages [99]. Under consideration of the growing body of knowledge concerning RING domain-containing ubiquitin ligases, investigation of ECTV and VARV p28 proteins showed that p28 acts as an ubiquitin ligase [100]. The respective gene is highly conserved among VARV, MPXV, CPXV, and ECTV, but inactivated in the known VACV strains. The molecular target (viral or cellular protein) of orthopoxvirus ubiquitin ligase p28 has not been identified yet.

Thus, orthopoxviruses possess at least seven genes encoding apoptosis inhibitors with very diverse modes of action (Table 4). This observation further confirms the importance of apoptosis in the mammalian system of antiviral defense.

5. Viral Interferon Inhibitors

Mammalian cells respond to viral infection by producing interferons (IFNs). The initial production of type I IFNs is due to activation of IFN regulatory factors (IRFs), and in particular IRF3, downstream of PRRs, which recognize viral DNA, RNA, and proteins [33]. It was discovered that some orthopoxviral Bcl-2-like proteins inhibit PRR-induced activation of IRFs (see Section 2 and Table 1) and therefore suppress IFN production.

IFNs are produced and secreted by animal cells also in response to double-stranded RNA molecules (dsRNA) synthesized in the course of viral infection. IFNs bind to specific cell receptors and induce antiviral defense state [101]. IFN-induced antiviral cell state is determined by at least two enzymatic pathways. One of them involves IFN-induced dsRNA-activated protein kinase (PKR); another one depends on 2-5A[ppp(A2′p)nA] synthetase (usually termed 2-5A synthetase). The protein kinase is activated by autophosphorylation, which occurs after protein binding to dsRNA. The activated PKR phosphorylates subunit alpha of the eukaryotic translation initiation factor (eIF-2α), thus blocking protein synthesis. The other enzyme, 2-5A synthetase is activated by dsRNA and catalyzes ATP polymerization to 2′-5′oligoadenylates, which, in turn, activate latent cellular endo-RNase L. RNase L cleaves mRNA and rRNA molecules, thus also disturbing protein synthesis.

Although orthopoxviruses produce high levels of virus-specific dsRNA in the late phase of their life cycle [102], they are highly resistant to IFN action [103]. VACV gene *E3L* encodes an inhibitor of the IFN-induced PRK [104]. This viral protein produced directly after cell infection can bind to dsRNA, competing with the specific cellular protein kinase and preventing the enzyme activation. Another VACV gene, *K3L*, encodes an eIF-2α homologue competing with endogenous eIF-2α for phosphorylation by activated PRK [105]. A mutant VACV strain with disrupted *K3L* is interferonsensitive and produces two orders of magnitude less viral progeny [106]. Thus, orthopoxviruses produce proteins that inhibit the activity of the IFN-induced PRK in two independent ways.

It should be noted that although the sequences of viral eIF-2α homologues are well conserved within species, the VARV protein has numerous differences in amino acids from highly homologous VACV and CPXV isologs, while MPXV

Table 4: Orthopoxviral proteins modulating defense reactions of mammals.

Protein function	VACV-COP		CPXV-GRI		MPXV-ZAI		VARV-IND	
	ORF	Size, aa	ORF	Size, aa	ORF	Size, aa	ORF	Size, aa
Apoptosis inhibitor, caspase-1 and caspase-8 inhibitor, SPI-2	**B13R**	**116**	B12R	345	B12R	344	B13R	344
Apoptosis inhibitor, SPI-1	C12L	353	B20R	375	B19R	357	B25R	372
Apoptosis inhibitor, Bcl-2-like	N1L	117	Q1L	117	P1L	117	P1L	117
Mitochondria-associated apoptosis inhibitor, caspase-9 inhibitor	F1L	226	G1L	238	C7L	219	C5L	251
Apoptosis inhibitor, transmembrane protein of Golgi apparatus	**None**	**None**	T1R	210	**R1R**	**105**	**None**	**None**
Apoptosis inhibitor, RING-domain containing E3 ubiquitin ligase	**None**	**None**	C7R	242	D5R	242	D4R	242
Apoptosis inhibitor, dsRNA-binding, interferon resistance	E3L	190	F3L	190	**F3L**	**153**	E3L	190
eIF-2α homolog, interferon resistance	K3L	88	M3L	88	**None**	**None**	C3L	88
Phosphatase, dephosphorylation of Stat 1	H1L	171	J1L	171	H1L	171	I1L	171
γ-IFN-binding	B8R	272	B7R	271	B9R	267	B9R	266
α/β-IFN-binding	B19R	353	B17R	351	B16R	352	B20R	354
IL-1β-binding	**None**	**None**	C8L	124	D6L	126	D5L	126
IL-18-binding	**B16R**	**290**	B14R	326	B14R	326	**B15R**	**63**
Complement binding	C3L	263	C17L	259	**D14L**	**216**	D12L	263
TNF- and chemokine-binding, CrmB	**B28R**	**122**	H4R	351	J2R	348	G2R	349
TNF-binding, CrmC	**A53R**	**103**	A56R	186	**None**	**None**	**None**	**None**
TNF- and chemokine-binding, CrmD	**None**	**None**	K2R	322	**None**	**None**	**None**	**None**
TNF-binding, CrmE	**None**	**None**	K3R	167	**K1R**	**70**	**None**	**None**
CC-chemokine-binding	**B29R**	**244**	I5R	255	J3R	246	G3R	253
CC- и CXC-chemokine-binding	A41L	219	A43L	219	A41L	221	A46L	218
Inhibitor of NK-mediated NKG2D-dependent lysis of infected cells	**None**	**None**	C2L	178	N3R	176	**None**	**None**
Inhibitor of MHC class II antigen presentation	A35R	176	A36R	176	A37R	176	**A38R**	**60**
Inhibitor of MHC class I complexes release from the PLC	**None**	**None**	D10L	96	**None**	**None**	**None**	**None**
Inhibitor of the intracellular trafficking of MHC class I molecules	**B9R**	**77**	B8R	221	B10R	221	**None**	**None**

ORFs that are altered/nonfunctional as compared with a CPXV-GRI counterpart are set in bold.

does not encode this interferon resistance factor due to multiple mutations of the respective gene (Table 4).

In the course of VACV infection, *E3L* is expressed from the first and the second initiator codon producing the long and the short protein forms, respectively [106]. The N-terminal domain of the long form is required for the protein binding to Z-form DNA, which explains its nuclear localization and its pathogenic properties [107, 108]. The C-terminal domain of both the long and the short form binds dsRNA and inhibits the activation of the PRK [109] and 2-5A synthetase [110]. In MPXV-ZAI, the first initiator triplet is disrupted by a mutation; for this reason, only the short protein form is translated. Thus, MPXV differs from other orthopoxvirus species in the unique organization of viral intracellular interferon resistance factors [64, 111], which apparently results in decreased propagation rates *in vivo* and, consequently, decreased efficiency of airborne transmission of the virus, which is indeed the case for human monkeypox, as compared to smallpox.

Recently, it has been demonstrated that VACV E3 protein also inhibits the type III λ-IFN-mediated antiviral response [112].

Blocking the function of Stat (signal transducer and activator of transcription) proteins, which are critical for antiviral responses, has evolved as a common mechanism for pathogen immune evasion. The VACV-encoded phosphatase H1 is critical for virus replication and plays an additional role in the evasion of host defense by dephosphorylating Stat1 and blocking IFN-stimulated innate immune responses. It was demonstrated that VARV H1 isolog (I1 for VARV-IND, see Table 4) is more active than VACV H1 in Stat1 dephosphorylation [113].

A unique and efficient IFN evasion strategy employed additionally by poxviruses is to encode soluble proteins that

are secreted from infected cells and function as soluble IFN decoy receptors. These factors providing interferon resistance in orthopoxviruses are extracellular γ-IFN-binding protein [114] and type I α/β-IFN-binding protein (α/β-IFN-BP) [115]. Earlier we have revealed pronounced species-specific differences in amino acid sequences of α/β-IFN-BPs of orthopoxviruses [3, 116]. Recently it has been shown that the VARV α/β-IFN-BP binds the human ligands with higher affinity than the VACV α/β-IFN-BP [117].

Thus, orthopoxviruses possess a multigene system providing a high level of interferon resistance. Differences found among these genes/proteins in VARV, MPXV, CPXV, and VACV (Table 4) call for further investigation of their properties.

6. Viral Inhibitors of Inflammatory Response

Inflammatory reactions play an important role in the early nonspecific protection of the organism against the viral infection. They are induced rapidly to limit the virus dissemination during the first hours and days upon infection while the full-fledged adaptive immune response is being formed. It is known that the complement system and the cytokines, such as tumor necrosis factor (TNF), interleukin-1β (IL-1β), gamma-interferon (γ-IFN), and chemokines, play the key role in inducing the inflammatory reactions [118]. In addition, several other mediators influence either directly or indirectly the development of the inflammatory process [116, 119–121]. Therefore, poxviruses potentially need several genes whose protein products are able to act as inhibitors of various stages of inflammation development to suppress efficiently the inflammatory response.

The first viral gene whose product represses inflammatory response to infection was found in CPXV and termed SPI-2 (*B12R* for CPXV-GRI, see Table 4) [122]. As noted above (see Section 3), SPI-2 inhibits caspase 1 activity and thus prevents the processing of pro-IL-1β to IL-1β and its secretion from the infected cell, suppressing, as a result, the induction of local inflammatory reactions. In addition, SPI-2 inhibits the production of inflammatory mediators (leukotrienes) in the arachidonic acid metabolism [123]. Furthermore, as discussed above, SPI-2 is also involved in suppressing apoptosis of the infected cell. Thus, this protein evidently plays an important role in determining orthopoxvirus pathogenicity *in vivo*. It should be noted that amino acid sequences of SPI-2 variants present in VARV, MPXV, and CPXV are somewhat different [32]. In the case of VACV-COP, the gene encoding this protein is damaged (Table 4).

It was shown experimentally that the VACV-WR gene *B15R* encodes a secreted glycoprotein acting as a soluble IL-1β receptor [124]. The production of this soluble receptor prevents the development of systemic reactions (such as fever) in VACV-infected mice [125]. It was shown that a VACV-WR strain with disrupted *B15R* had increased virulence in mice (when administered intranasally) [124]. A further analysis showed that VACV strains associated with a higher frequency of postvaccination complications in humans lack IL-1β-binding activity [125]. These data agree well with the fact that the respective gene in VARV is disrupted (by fragmentation) (Table 4).

Thus, we may hypothesize that VARV suppresses production and secretion of IL-1β by infected cells but does not inhibit the effect of extracellular IL-1β synthesized by other cells of the body. This suggests that VARV is capable of suppressing local inflammatory reactions due to SPI-2 production in the region of virus replication; however, it does not inhibit the systemic reactions, as it is unable to synthesize IL-1β-binding protein. Decrease in the local inflammatory reactions may assist a more active virus replication, while uncontrolled development of the systemic reactions weakens the overall resistance of the organism to infection. A concurrent development of these reactions is likely to boost the pathogenic effect of the viral infection on the host organism. In the case of MPXV and CPXV, both genes in question are native (Table 4).

Orthopoxviruses, in particular, VACV-WR, but not the less virulent VACV-COP strain, also encode an IL-18-binding protein (Table 4), which is secreted from the cell and suppresses the activity of proinflammatory IL-18 [126].

Similarly to other cytokines, TNF performs multiple functions [118]. In particular, as noted above, it is a key cytokine inducing inflammation in the infected host along with IL-1β and IL-18. It was shown that VARV-IND gene *G2R* encodes CrmB protein homologous to type II TNF receptor [127]. An orthologous TNF-inhibitory protein called M-T2 is an important secreted virulence factor of the rabbit myxoma virus (a poxvirus of the genus *Leporipoxvirus*). Its VARV analogue G2 apparently has similar properties. An important difference between VARV and VACV is that the latter possesses no genes encoding TNF receptor analogues. In the CPXV genome, we detected five genes of the TNF receptor family [23]. Four of them have TNF-binding activity [128–131] (Table 4).

An analysis of amino acid sequences of CrmB isologs detected numerous species-specific differences. Using a baculuvirus expression system, we obtained individual CrmB proteins of VARV, MPXV, and CPXV and showed that their ability to suppress the activity of human, mouse, and rabbit TNFs differs considerably. Only CrmB-VARV inhibits human TNF activity with high efficiency [132, 133]. Presumably, this is a result of evolutionary adaptation of the viral receptors to the ligands of their hosts.

It was recently shown that orthopoxvirus TNF-binding protein CrmB possesses a further biological activity; that is, it has high affinity to certain chemokines critically involved in attracting dendritic cells, B-, and T-lymphocytes to the inflammation focus [134]. Its immunomodulatory activity is determined by the unique C-terminal domain termed SECRET (smallpox virus-encoded chemokine receptor). The amino acid sequence of this domain has no homology to any vertebrate protein or to any other known viral chemokine-binding protein. *De novo* modeling of the spatial structure of the SECRET domain showed that it might be a structural homologue of the secreted CC-chemokine-binding protein G3 of VARV (Table 4), in spite of the low similarity of their amino acid sequences [135].

Chemokines are chemoattractant cytokines, which control migration and effector functions of leukocytes, thereby playing an important role in development of inflammatory response and protection against pathogens [136]. It was demonstrated that VACV strain Lister at the early stages of infection produced a protein secreted from the cells in large amounts [137], which bound a wide range of CC chemokines and inhibited their activities [138]. This gene is damaged in many other VACV strains. Presumably, isologs of this protein (G3 in VARV-IND) of various orthopoxvirus species have different functions, as analysis of their amino acid sequences detected considerable species-specific distinctions [3].

The VACV protein A41 and its orthopoxvirus isologs also are secreted glycoproteins that efficiently and selectively bind to certain CC and CXC chemokines preventing chemokine-induced leukocyte migration to the infection locus [139, 140]. This chemokine-binding protein probably is essential for virus propagation, since it is conserved in all orthopoxvirus species studied (Table 4).

Interestingly, all orthopoxviruses in question also produce a soluble γ-IFN-receptor, which can modulate the host's inflammatory response to infection [141–143]. The protein B9 of VARV-IND and its isolog produced by VACV-COP contain a considerable number of amino acid substitutions [144]. Probably, these species-specific differences in the structure of viral γ-IFN-binding protein are related to the difference in VARV and VACV virulence.

In addition to the above genes, orthopoxviruses also carry a gene of a complement-binding protein (*C3L* in VACV-COP) [145], one of whose functions may be regulation of inflammation. The complement system comprises over 20 blood plasma proteins. Antiviral functions of the complement systems include virus neutralization, lysis of infected cells, and enhancement of inflammatory and adaptive immune response [2, 119].

The VACV protein C3, named VCP, secreted from infected cells and controlling the reaction of complement activation comprises four short degenerated repeats of approximately 60 amino acids each (short consensus repeat, SCR) characteristic of the protein family of complement activation regulators (RCA) [146]. It is considered that the gene encoding VCP originated initially due to incorporating a part or the complete coding sequence of a protein belonging to RCA family of the host into the viral genome followed by adaptation (alteration) of the gene in question to perform the functions necessary for the virus [147]. The X-ray structural analysis showed that SCR sequences of VCP form a series of discrete tightly linked compact domains [148].

VCP is a unique multifunctional viral protein functionally resembling as different RCA proteins as factor H, membrane-bound cofactor protein, type I complement receptor, and decay-accelerating factor (DAF). Firstly, VCP binds complement components C3b and C4b; secondly, it blocks different stages of the complement cascade and inhibits both the classical and the alternative complement pathways; thirdly, it blocks complement-driven virus neutralization activated by antiviral antibodies, and, finally, binds heparin-like molecules on the surface of endothelial cells, blocking the binding of chemokines and preventing signal transduction for chemotaxis [119]. The model of CPXV-infected mice showed that VCP suppresses inflammatory response *in vivo* [149, 150].

VCPs of VARV, CPXV, and VACV contain four SCRs each. We have revealed the unique structure of the MPXV VCP [3, 151]. Due to premature termination of synthesis, the protein sequence is truncated and the C-terminal SCR-4 is deleted in Central African MPXV strains, whereas Western African MPXV strains lack the gene for VCP completely [3]. Possibly this deletion or truncation of the gene for VCP prevents effective inhibition of inflammatory response by MPXV and therefore the specific feature of human monkeypox clinical course, distinguishing it from smallpox, is lymphadenitides.

Amino acid sequences of VACV and VARV VCPs differ at 12 positions. A baculovirus system was used to produce individual VCPs of VARV and VACV [152]. It was shown that VCP of VARV is a significantly more efficient inhibitor of human complement than its VACV counterpart. This observation further supports the concept that viral soluble receptors are evolutionary adapted to the host's ligands.

To sum up, orthopoxviruses possess a multigene system controlling at different stages development of the host's inflammatory reactions. Orthopoxviruses display species-specific distinctions not only in the set of these genes but also in their structure, and as a result in targeted activities of the encoding proteins.

7. Orthopoxvirus Modulation of Cellular Immune Response

One of the principal mechanisms of the innate cellular immunity involves nonspecific lysis of virus-infected cells by natural killer (NK) cells [153]. The latter are activated by soluble mediators or in a direct cell contact. NK proliferation peaks on days 2-3 of orthopoxvirus infection; however, these cells alone are unable to prevent completely the dissemination of infection in the body [154]. NK cell activation is regulated by integrated signals of several activating and suppressing receptors, many of which use major histocompatibility complex (MHC) class I molecules or related proteins as ligands. One of the cytotoxic NK-activating receptors is NKG2D. It was shown that CPXV and MPXV encode a protein resembling an MHC class I molecule (OMCP; C2 in CPXV-GRI) that blocks the recognition of host ligands and inhibits the NKG2D-dependent NK lysis of infected cells [155]. VARV and VACV genomes lack such a gene (Table 4).

As noted above, orthopoxviruses produce a secreted IL-18-binding protein, which blocks not only the proinflammatory activity of IL-18 but also IL-18 induced NK cytotoxicity [126, 156].

Adaptive immune response to infection involves complex cytokine-regulated interactions among different types of cells [157] that give rise to B-lymphocytes producing virus-specific antibodies and virus-specific cytolytic T lymphocytes. Proliferation of virus-specific cytolytic T lymphocytes

peaks on days 5-6 of infection; the key regulatory cytokines TNF, IL-1β, and γ-IFN control not only inflammatory reactions but also the adaptive immune response. Specific antibodies can interact with virus particles and their components individually or within complement complexes. Specific antibodies are inefficient in controlling primary poxvirus infection but can be important in preventing secondary infection [154]. Cellular immune response is a crucial component of specific defense against a poxvirus infection [158].

For some orthopoxviruses, it has been shown that they directly infect human and rodent immune cells both *in vitro* and *in vivo*, including lymphocytes, NK cells, and monocytes/macrophages and VACV decrease antigen presentation in several types of antigen-presenting cells (APC) [159]. Recently, it was revealed that VACV-COP protein A35 inhibits MHC class II-restricted antigen presentation, immune priming of T lymphocytes, and subsequent cytokine and chemokine synthesis [160]. The gene of this protein is highly conservative for VACV, CPXV, and MPXV but in the genome of VARV is disrupted (see Table 4). Interestingly, for VACV MVA strain it was shown that deletion of the *A35R* gene increases its immunogenicity [161]. In this respect, it should be noted known data that the persons that had smallpox acquire a lifelong immunity, whereas the vaccination with VACV requires repeated immunizations with a certain periodicity to provide a reliable protection against smallpox [10]. We may speculate that VARV nonfunctional short *A38R* originated as a result of mutational changes in functional *A35R* analog of ancestral zoonotic orthopoxvirus and it allowed to highly virulent VARV caused lifelong immunity in humans and provided the additional conditions for smallpox endemization [4].

MHC class I molecules play an important role in antiviral immunity. The majority of MHC class I-binding peptides are generated in the cytosol by proteasomes and transported into the lumen of the endoplasmic reticulum (ER) by transporter associated with antigen processing (TAP). Peptide loading onto the MHC class I heavy chain-β2 m heterodimer is facilitated by a multi-subunit protein complex called the MHC class I peptide-loading complex (PLC). In addition to TAP and MHC class I, the PLC is composed of ER chaperones. Upon peptide loading, the fully assembled MHC class I complexes dissociate from the PLC and transit to the cell surface. Recognition of viral peptides in the context of MHC class I molecules triggers virus-specific CD8 T cells to exert their effector functions including cytotoxicity and cytokine secretion [162].

Recently, it has been shown that CPXV downregulates MHC class I and evades antiviral CD8 T cell responses [163]. Two distinctly acting MHC class I regulating genes (*D10L* and *B8R* for CPXV-GRI, see Table 4) have been revealed. Protein D10 inhibits MHC class I expression by impairing ER peptide loading and dissociation of MHC class I from TAP. Protein B8 interferes with the intracellular trafficking of MHC class I molecules by sequestering them in the ER using its C-terminal KDEL-like sequence [162].

The *in vivo* significance of the discovered viral MHC class I and class II evasion mechanisms, however, is not well understood. Among orthopoxviruses pathogenic for humans only CPXV produces all three known regulators of MHC systems. Highly virulent and highly immunogenic for humans, VARV does not produce any of these proteins (Table 4).

8. Conclusion

Comparison of amino acid sequences of a great number of various types of human and rodent polypeptides revealed most pronounced interspecies differences in the sequences of the proteins forming ligand-receptor pairs of the organismal protective systems of these mammals against infectious agents. In addition, the polypeptide ligands and their receptors proved to be subjected to coevolution [164]. Pathogenic microorganisms are assumed to be able to cause an accelerated evolution of the defense system proteins (genes) of infected animal species. Such evolutionary changes in the primary structure of the proteins constituting ligand-receptor pairs were suggested to result in alterations of the quaternary structure of the ligand-receptor contact region [164, 165]. As a result, the species-specific mimicry of mammalian defense system proteins may emerge, providing a narrower range of hosts sensitive to certain infectious microorganism [164].

The virus that maintains the balance between its pathogenic effect on the host organism and the possibility of its effective development in the animal organism for a relatively long period is the best adapted from the evolutionary standpoint. Such virus is able to transmit efficiently from animal to animal under a low population density. Among orthopoxviruses, CPXV most pronouncedly displays such properties. It is worth noting that CPXV encodes the complete set of immunomodulatory (Table 4) as well as Bcl-2-like (Table 1), ankyrin-like (Table 2), and kelch-like (Table 3) proteins found in orthopoxviruses, whereas VARV, MPXV, and VACV each possess an incomplete species-specific subset of these genes.

The species- and strain-specific distinctions between VARV, MPXV, CPXV, and VACV DNAs are localized to the long variable terminal regions [3, 23, 63, 64, 166–168]. These distinctions comprise not only deletions in DNAs of the viruses compared relative one another but also rearrangements and nucleotide substitutions [3, 169]. The determined sequences of viral DNAs allow for a comparative analysis of organization of the VARV, MPXV, CPXV, and VACV molecular pathogenicity factors, whose function was verified at various laboratories in experiments mainly with VACV, CPXV, and ECTV [3, 22, 56, 116, 120, 121, 154, 157].

The CPXV genome has the largest size as compared with the other orthopoxviruses and contains the complete set of all the genes characteristic of other viruses from the genus *Orthopoxvirus* (Tables 1, 2, 3, and 4). The fact that CPXV nonetheless does not display an increased virulence suggests that orthopoxviruses have a certain regulatory system(s). To describe this regulatory system, we earlier introduced the concept of *buffer* genes, whose role is to neutralize the negative effects developing in the body during infection [22]. Presumably, CPXV possesses the widest set of these

genes as compared with VARV, MPXV, and VACV. The rest orthopoxvirus species have lost certain part of the genes with reference to CPXV. Note that VARV contains the shortest orthopoxviral genome and the least set of actual genes. This observation suggests that CPXV is most close to the ancestor of orthopoxviruses, while the rest species emerged later due to deletions, recombinations, and mutations [3, 5, 6, 169].

Humans are the only VARV host; hence, this virus is to a maximal degree adapted evolutionarily to overcome the human defense reactions, which develop in response to the infection. MPXV, CPXV, and VACV have wide host range, infecting first and foremost various rodents. Humans are sporadically infected by these viruses. Consequently, MPXV, CPXV, and VACV are adapted to interactions with the molecular defense reactions of mammals of various species [3, 4, 11, 13, 19, 21]. As we can see from the above-mentioned in these review cases, studied immunomodulatory proteins of VARV, as a rule, more effectively inhibit activities of their human ligands as compared with other species of orthopoxviruses pathogenic for humans. We may speculate that it is one of the most likely reasons of high VARV virulence for humans.

The interaction network of cytokines and their receptors has been so far studied only to a first approximation, and many discoveries are still awaiting researchers in this direction. Orthopoxviruses can play an important role here.

Summing up the available data, we may infer that VARV and other orthopoxviruses possess an unexampled set of genes whose protein products efficiently modulate the manifold defense functions of the host organisms compared with the viruses from other families. It is likely that by the example of orthopoxviruses, it will be possible in the nearest future to trace the patterns of coevolution of the viral pathogenicity factors and mammalian systems providing defense against infectious agents. The research into application of immunomodulatory proteins of orthopoxviruses, and first and foremost, variola virus, as drugs also deserves attention.

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References

[1] V. A. K. Rathinam and K. A. Fitzgerald, "Innate immune sensing of DNA viruses," *Virology*, vol. 411, no. 2, pp. 153–162, 2011.

[2] K. A. Stoermer and T. E. Morrison, "Complement and viral pathogenesis," *Virology*, vol. 411, no. 2, pp. 362–373, 2011.

[3] S. N. Shchelkunov, S. S. Marennikova, and R. W. Moyer, *Orthopoxviruses Pathogenic for Humans*, Springer, Berlin, Germany, 2005.

[4] S. N. Shchelkunov, "Emergence and reemergence of smallpox: the need in development of a new generation smallpox vaccine," *Vaccine*, vol. 29, supplement 4, pp. D49–D53, 2011.

[5] S. N. Shchelkunov, "How long ago did smallpox virus emerge?" *Archives of Virology*, vol. 154, no. 12, pp. 1865–1871, 2009.

[6] I. V. Babkin and S. N. Shchelkunov, "Time scale of poxvirus evolution," *Molecular Biology*, vol. 40, no. 1, pp. 16–19, 2006.

[7] B. Moss et al., "Poxviridae: the viruses and their replication," in *Fields Virology*, D. M. Knipe, P. M. Howley, D. E. Griffin et al., Eds., pp. 2905–2946, Lippincott Williams and Wilkins, Philadelphia, Pa, USA, 2007.

[8] D. J. Esteban and R. M. L. Buller, "Ectromelia virus: the causative agent of mousepox," *Journal of General Virology*, vol. 86, no. 10, pp. 2645–2659, 2005.

[9] J. L. Chapman, D. K. Nichols, M. J. Martinez, and J. W. Raymond, "Animal models of orthopoxvirus infection," *Veterinary Pathology*, vol. 47, no. 5, pp. 852–870, 2010.

[10] F. Fenner, D. A. Henderson, I. Arita, Z. Jezek, and I. D. Ladnyi, *Smallpox and Its Eradication*, World Health Organization, Geneva, Switzerland, 1988.

[11] J. G. Breman, "Monkeypox: an emerging infection of humans?" in *Emerging Infections 4*, W. M. Scheid, W. A. Craig, and J. M. Hughes, Eds., pp. 45–67, ASM Press, Washington, DC, USA, 2000.

[12] A. W. Rimoin, P. M. Mulembakani, S. C. Johnston et al., "Major increase in human monkeypox incidence 30 years after smallpox vaccination campaigns cease in the Democratic Republic of Congo," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 37, pp. 16262–16267, 2010.

[13] R. M. Vorou, V. G. Papavassiliou, and I. N. Pierroutsakos, "Cowpox virus infection: an emerging health threat," *Current Opinion in Infectious Diseases*, vol. 21, no. 2, pp. 153–156, 2008.

[14] H. Campe, P. Zimmermann, K. Glos et al., "Cowpox virus transmission from pet rats to humans, Germany," *Emerging Infectious Diseases*, vol. 15, no. 5, pp. 777–780, 2009.

[15] L. Ninove, Y. Domart, C. Vervel et al., "Cowpox virus transmission from pet rats to humans, France," *Emerging Infectious Diseases*, vol. 15, no. 5, pp. 781–784, 2009.

[16] S. Blackford, D. L. Roberts, and P. D. Thomas, "Cowpox infection causing a generalized eruption in a patient with atopic dermatitis," *British Journal of Dermatology*, vol. 129, no. 5, pp. 628–629, 1993.

[17] P. M. Pelkonen, K. Tarvainen, A. Hynninen et al., "Cowpox with severe generalized eruption, Finland," *Emerging Infectious Diseases*, vol. 9, no. 11, pp. 1458–1461, 2003.

[18] C. P. Czerny, A. M. Eis-Hübinger, A. Mayr, K. E. Schneweis, and B. Pfeiff, "Animal poxviruses transmitted from cat to man: current event with lethal end," *Zentralblatt fur Veterinarmedizin B*, vol. 38, no. 6, pp. 421–431, 1991.

[19] R. K. Singh, M. Hosamani, V. Balamurugan, V. Bhanuprakash, T. J. Rasool, and M. P. Yadav, "Buffalopox: an emerging and re-emerging zoonosis," *Animal Health Research Reviews*, vol. 8, no. 1, pp. 105–114, 2007.

[20] A. T. Silva-Fernandes, C. E. P. F. Travassos, J. M. S. Ferreira et al., "Natural human infections with vaccinia virus during bovine vaccinia outbreaks," *Journal of Clinical Virology*, vol. 44, no. 4, pp. 308–313, 2009.

[21] J. S. Abrahão, M. I. M. Guedes, G. S. Trindade et al., "One more piece in the VACV ecological puzzle: could peridomestic rodents be the link between wildlife and bovine vaccinia outbreaks in Brazil?" *PLoS ONE*, vol. 4, no. 10, Article ID e7428, 2009.

[22] S. N. Shchelkunov, "Functional organization of variola major and vaccinia virus genomes," *Virus Genes*, vol. 10, no. 1, pp. 53–71, 1995.

[23] S. N. Shchelkunov, P. F. Safronov, A. V. Totmenin et al., "The genomic sequence analysis of the left and right

species-specific terminal region of a cowpox virus strain reveals unique sequences and a cluster of intact ORFs for immunomodulatory and host range proteins," *Virology*, vol. 243, no. 2, pp. 432–460, 1998.

[24] T. Kawai and S. Akira, "The role of pattern-recognition receptors in innate immunity: update on toll-like receptors," *Nature Immunology*, vol. 11, no. 5, pp. 373–384, 2010.

[25] M. Lamkanfi and V. M. Dixit, "The inflammasomes," *PLoS Pathogens*, vol. 5, no. 12, Article ID e1000510, 2009.

[26] A. A. Abdul-Sater, N. Saïd-Sadier, D. M. Ojcius, O. Yilmaz, and K. A. Kelly, "Inflammasomes bridge signaling between pathogen identification and the immune response," *Drugs of Today*, vol. 45, pp. 105–112, 2009.

[27] J. H. Pedra, S. L. Cassel, and F. S. Sutterwala, "Sensing pathogens and danger signals by the inflammasome," *Current Opinion in Immunology*, vol. 21, no. 1, pp. 10–16, 2009.

[28] A. Bowie, E. Kiss-Toth, J. A. Symons, G. L. Smith, S. K. Dower, and L. A. J. O'Neill, "A46R and A52R from vaccinia virus are antagonists of host IL-1 and toll-like receptor signaling," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 18, pp. 10162–10167, 2000.

[29] M. T. Harte, I. R. Haga, G. Maloney et al., "The poxvirus protein A52R targets toll-like receptor signaling complexes to suppress host defense," *Journal of Experimental Medicine*, vol. 197, no. 3, pp. 343–351, 2003.

[30] J. Stack, I. R. Haga, M. Schröder et al., "Vaccinia virus protein A46R targets multiple Toll-like-interleukin-1 receptor adaptors and contributes to virulence," *Journal of Experimental Medicine*, vol. 201, no. 6, pp. 1007–1018, 2005.

[31] S. C. Graham, M. W. Bahar, S. Cooray et al., "Vaccinia virus proteins A52 and B14 share a Bcl-2-like fold but have evolved to inhibit Nf-κB rather than apoptosis," *PLoS Pathogens*, vol. 4, no. 8, Article ID e1000128, 2008.

[32] J. M. Gonzlez and M. Esteban, "A poxvirus Bcl-2-like gene family involved in regulation of host immune response: sequence similarity and evolutionary history," *Virology Journal*, vol. 7, article 59, 2010.

[33] L. Unterholzner, R. P. Sumner, M. Baran et al., "Vaccinia virus protein C6 is a virulence factor that binds TBK-1 adaptor proteins and inhibits activation of IRF3 and IRF7," *PLoS Pathogens*, vol. 7, no. 9, Article ID 100224, 2011.

[34] C. M. de Motes, S. Cooray, H. Ren et al., "Inhibition of apoptosis and NF-êB activation by vaccinia protein N1 occur via distinct binding surfaces and make different contributions to virulence," *PLoS Pathogens*, vol. 7, no. 12, Article ID 100243, 2011.

[35] A. Ciechanover, "Intracellular protein degradation: from a vague idea thru the lysosome and the ubiquitin-proteasome system and onto human diseases and drug targeting," *Experimental Biology and Medicine*, vol. 231, no. 7, pp. 1197–1211, 2006.

[36] D. R. Bosu and E. T. Kipreos, "Cullin-RING ubiquitin ligases: global regulation and activation cycles," *Cell Division*, vol. 3, article e7, 2008.

[37] M. Hochstrasser, "Origin and function of ubiquitin-like proteins," *Nature*, vol. 458, no. 7237, pp. 422–429, 2009.

[38] E. T. Kipreos, L. E. Lander, J. P. Wing, W. W. He, and E. M. Hedgecock, "cul-1 is required for cell cycle exit in *C. elegans* and identifies a novel gene family," *Cell*, vol. 85, no. 6, pp. 829–839, 1996.

[39] C. A. P. Joazeiro and A. M. Weissman, "RING finger proteins: mediators of ubiquitin ligase activity," *Cell*, vol. 102, no. 5, pp. 549–552, 2000.

[40] E. T. Kipreos and M. Pagano, "The F-box protein family," *Genome Biology*, vol. 1, no. 5, pp. reviews3002–reviews 3002.7, 2000.

[41] J. Jin, T. Cardozo, R. C. Lovering, S. J. Elledge, M. Pagano, and J. W. Harper, "Systematic analysis and nomenclature of mammalian F-box proteins," *Genes and Development*, vol. 18, no. 21, pp. 2573–2580, 2004.

[42] L. Pintard, A. Willems, and M. Peter, "Cullin-based ubiquitin ligases: Cul3-BTB complexes join the family," *The EMBO Journal*, vol. 23, no. 8, pp. 1681–1687, 2004.

[43] P. J. Stogios, G. S. Downs, J. J. Jauhal, S. K. Nandra, and G. G. Privé, "Sequence and structural analysis of BTB domain proteins," *Genome Biology*, vol. 6, no. 10, article R82, 2005.

[44] M. Furukawa and Y. Xiong, "BTB protein keap1 targets antioxidant transcription factor Nrf2 for ubiquitination by the cullin 3-Roc1 ligase," *Molecular and Cellular Biology*, vol. 25, no. 1, pp. 162–171, 2005.

[45] G. Gao and H. Luo, "The ubiquitin-proteasome pathway in viral infections," *Canadian Journal of Physiology and Pharmacology*, vol. 84, no. 1, pp. 5–14, 2006.

[46] M. J. Edelmann and B. M. Kessler, "Ubiquitin and ubiquitin-like specific proteases targeted by infectious pathogens: emerging patterns and molecular principles," *Biochimica et Biophysica Acta*, vol. 1782, no. 12, pp. 809–816, 2008.

[47] H. A. Lindner, "Deubiquitination in virus infection," *Virology*, vol. 362, no. 2, pp. 245–256, 2007.

[48] P. Blanchette and P. E. Branton, "Manipulation of the ubiquitin-proteasome pathway by small DNA tumor viruses," *Virology*, vol. 384, no. 2, pp. 317–323, 2009.

[49] N. Tolonen, L. Doglio, S. Schleich, and J. Krijnse Locker, "Vaccinia virus DNA replication occurs in endoplasmic reticulum-enclosed cytoplasmic mini-nuclei," *Molecular Biology of the Cell*, vol. 12, no. 7, pp. 2031–2046, 2001.

[50] A. Teale, S. Campbell, N. Van Buuren et al., "Orthopoxviruses require a functional ubiquitin-proteasome system for productive replication," *Journal of Virology*, vol. 83, no. 5, pp. 2099–2108, 2009.

[51] P. S. Satheshkumar, L. C. Anton, P. Sanz, and B. Moss, "Inhibition of the ubiquitin-proteasome system prevents vaccinia virus dna replication and expression of intermediate and late genes," *Journal of Virology*, vol. 83, no. 6, pp. 2469–2479, 2009.

[52] C. S. Chung, C. H. Chen, M. Y. Ho, C. Y. Huang, C. L. Liao, and W. Chang, "Vaccinia virus proteome: identification of proteins in vaccinia virus intracellular mature virion particles," *Journal of Virology*, vol. 80, no. 5, pp. 2127–2140, 2006.

[53] R. B. Luftig, "Does the cytoskeleton play a significant role in animal virus replication?" *Journal of Theoretical Biology*, vol. 99, no. 1, pp. 173–191, 1982.

[54] A. Schepis, B. Schramm, C. A. M. de Haan, and J. K. Locker, "Vaccinia virus-induced microtubule-dependent cellular rearrangements," *Traffic*, vol. 7, no. 3, pp. 308–323, 2006.

[55] P. M. Steinert and D. R. Roop, "Molecular and cellular biology of intermediate filaments," *Annual Review of Biochemistry*, vol. 57, pp. 593–625, 1988.

[56] F. Fenner, R. Wittek, and K. R. Dumbell, *The Orthopoxviruses*, Academic Press, New York, NY, USA, 1989.

[57] M. S. Hayden and S. Ghosh, "Shared principles in NF-κB signaling," *Cell*, vol. 132, no. 3, pp. 344–362, 2008.

[58] S. J. Chang, J. C. Hsiao, S. Sonnberg et al., "Poxvirus host range protein CP77 contains an F-box-like domain that is necessary to suppress NF-κB activation by tumor necrosis

factor alpha but is independent of its host range function," *Journal of Virology*, vol. 83, no. 9, pp. 4140–4152, 2009.

[59] J. L. Shisler and X. L. Jin, "The vaccinia virus K1L gene product inhibits host NF-κB activation by preventing IκBα degradation," *Journal of Virology*, vol. 78, no. 7, pp. 3553–3560, 2004.

[60] S. E. Lux, K. M. John, and V. Bennett, "Analysis of cDNA for human erythrocyte ankyrin indicates a repeated structure with homology to tissue-differentiation and cell-cycle control proteins," *Nature*, vol. 344, no. 6261, pp. 36–42, 1990.

[61] S. Lambert, H. Yu, J. T. Prchal et al., "cDNA sequence for human erythrocyte ankyrin," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 87, no. 5, pp. 1730–1734, 1990.

[62] S. N. Shchelkunov, V. M. Blinov, and L. S. Sandakhchiev, "Ankyrin-like proteins of variola and vaccinia viruses," *FEBS Letters*, vol. 319, no. 1-2, pp. 163–165, 1993.

[63] S. N. Shchelkunov, "Interaction of orthopoxviruses with the cellular ubiquitin-ligase system," *Virus Genes*, vol. 41, no. 3, pp. 309–318, 2010.

[64] S. N. Shchelkunov, A. V. Totmenin, I. V. Babkin et al., "Human monkeypox and smallpox viruses: genomic comparison," *FEBS Letters*, vol. 509, no. 1, pp. 66–70, 2001.

[65] C. Meisinger-Henschel, M. Schmidt, S. Lukassen et al., "Genomic sequence of chorioallantois vaccinia virus Ankara, the ancestor of modified vaccinia virus Ankara," *Journal of General Virology*, vol. 88, no. 12, pp. 3249–3259, 2007.

[66] A. A. Mercer, S. B. Fleming, and N. Ueda, "F-box-like domains are present in most poxvirus ankyrin repeat proteins," *Virus Genes*, vol. 31, no. 2, pp. 127–133, 2005.

[67] S. Sonnberg, B. T. Seet, T. Pawson, S. B. Fleming, and A. A. Mercer, "Poxvirus ankyrin repeat proteins are a unique class of F-box proteins that associate with cellular SCF1 ubiquitin ligase complexes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 31, pp. 10955–10960, 2008.

[68] S. Sonnberg, S. B. Fleming, and A. A. Mercer, "A truncated two-α-helix F-box present in poxvirus ankyrin-repeat proteins is sufficient for binding the SCF1 ubiquitin ligase complex," *Journal of General Virology*, vol. 90, no. 5, pp. 1224–1228, 2009.

[69] M. R. Mohamed, M. M. Rahman, J. S. Lanchbury et al., "Proteomic screening of variola virus reveals a unique NF-κB inhibitor that is highly conserved among pathogenic orthopoxviruses," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 22, pp. 9045–9050, 2009.

[70] K. M. Sperling, A. Schwantes, C. Staib, B. S. Schnierle, and G. Sutter, "The orthopoxvirus 68-kilodalton ankyrin-like protein is essential for DNA replication and complete gene expression of modified vaccinia virus ankara in nonpermissive human and murine cells," *Journal of Virology*, vol. 83, no. 12, pp. 6029–6038, 2009.

[71] N. van Buuren, B. Couturier, Y. Xiong, and M. Barry, "Ectromelia virus encodes a novel family of F-box proteins that interact with the SCF complex," *Journal of Virology*, vol. 82, no. 20, pp. 9917–9927, 2008.

[72] T. G. Senkevich, G. L. Muravnik, S. G. Pozdnyakov et al., "Nucleotide sequence of *Xho*I fragment of ectromelia virus DNA reveals significant differences from vaccinia virus," *Virus Research*, vol. 30, no. 1, pp. 73–88, 1993.

[73] S. N. Shchelkunov, V. M. Blinov, S. M. Resenchuk et al., "Analysis of the nucleotide sequence of 53 kbp from the right terminus of the genome of variola major virus strain India-1967," *Virus Research*, vol. 34, no. 3, pp. 207–236, 1994.

[74] S. Shchelkunov, A. Totmenin, and I. Kolosova, "Species-specific differences in organization of orthopoxvirus kelch-like proteins," *Virus Genes*, vol. 24, no. 2, pp. 157–162, 2002.

[75] N. Chen, M. I. Danila, Z. Feng et al., "The genomic sequence of ectromelia virus, the causative agent of mousepox," *Virology*, vol. 317, no. 1, pp. 165–186, 2003.

[76] M. E. Perkus, S. J. Goebel, S. W. Davis, G. P. Johnson, E. K. Norton, and E. Paoletti, "Deletion of 55 open reading frames from the termini of vaccinia virus," *Virology*, vol. 180, no. 1, pp. 406–410, 1991.

[77] B. A. Wilton, S. Campbell, N. Van Buuren et al., "Ectromelia virus BTB/kelch proteins, EVM150 and EVM167, interact with cullin-3-based ubiquitin ligases," *Virology*, vol. 374, no. 1, pp. 82–99, 2008.

[78] L. Zhang, N. Y. Villa, and G. McFadden, "Interplay between poxviruses and the cellular ubiquitin/ubiquitin-like pathways," *FEBS Letters*, vol. 583, no. 4, pp. 607–614, 2009.

[79] G. V. Kochneva, I. V. Kolosova, T. A. Lupan et al., "Orthopoxvirus genes for kelch-like proteins: III. Construction of mousepox (ectromelia) virus variants with targeted gene deletions," *Molecular Biology*, vol. 43, no. 4, pp. 567–572, 2009.

[80] G. Kochneva, I. Kolosova, T. Maksyutova, E. Ryabchikova, and S. Shchelkunov, "Effects of deletions of kelch-like genes on cowpox virus biological properties," *Archives of Virology*, vol. 150, no. 9, pp. 1857–1870, 2005.

[81] G. V. Kochneva, O. S. Taranov, T. A. Lupan et al., "Investigation of the impact of cowpox virus BTB/kelch gene deletion on some characteristics of infection *in vitro*," *Voprosy Virusologii*, vol. 54, no. 1, pp. 28–32, 2009.

[82] M. P. de Miranda, P. C. Reading, D. C. Tscharke, B. J. Murphy, and G. L. Smith, "The vaccinia virus kelch-like protein C2L affects calcium-independent adhesion to the extracellular matrix and inflammation in a murine intradermal model," *Journal of General Virology*, vol. 84, no. 9, pp. 2459–2471, 2003.

[83] P. M. Beard, G. C. Froggatt, and G. L. Smith, "Vaccinia virus kelch protein A55 is a 64 kDa intracellular factor that affects virus-induced cytopathic effect and the outcome of infection in a murine intradermal model," *Journal of General Virology*, vol. 87, no. 6, pp. 1521–1529, 2006.

[84] G. C. Froggatt, G. L. Smith, and P. M. Beard, "Vaccinia virus gene F3L encodes an intracellular protein that affects the innate immune response," *Journal of General Virology*, vol. 88, no. 7, pp. 1917–1921, 2007.

[85] J. F. Kerr, A. H. Wyllie, and A. R. Currie, "Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics," *British Journal of Cancer*, vol. 26, no. 4, pp. 239–257, 1972.

[86] M. R. Hilleman, "Strategies and mechanisms for host and pathogen survival in acute and persistent viral infections," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, supplement 2, pp. 14560–14566, 2004.

[87] E. Domingo-Gil and M. Esteban, "Role of mitochondria in apoptosis induced by the 2-5A system and mechanisms involved," *Apoptosis*, vol. 11, no. 5, pp. 725–738, 2006.

[88] M. G. Netea, A. Simon, F. van de Veerdonk, B. J. Kullberg, J. W. M. van der Meer, and L. A. B. Joosten, "IL-1β processing in host defense: beyond the inflammasomes," *PLoS Pathogens*, vol. 6, no. 2, Article ID e1000661, 2010.

[89] J. Macen, A. Takahashi, K. B. Moon, R. Nathaniel, P. C. Turner, and R. W. Moyer, "Activation of caspases in pig kidney cells infected with wild-type and CrmA/SPI-2 mutants of cowpox and rabbitpox viruses," *Journal of Virology*, vol. 72, no. 5, pp. 3524–3533, 1998.

[90] B. T. Seet, J. B. Johnston, C. R. Brunetti et al., "Poxviruses and immune evasion," *Annual Review of Immunology*, vol. 21, pp. 377–423, 2003.

[91] B. G. Luttge and R. W. Moyer, "Suppressors of a host range mutation in the rabbitpox virus serpin SPI-1 map to proteins essential for viral DNA replication," *Journal of Virology*, vol. 79, no. 14, pp. 9168–9179, 2005.

[92] J. M. Taylor, D. Quilty, L. Banadyga, and M. Barry, "The vaccinia virus protein F1L interacts with Bim and inhibits activation of the pro-apoptotic protein Bax," *Journal of Biological Chemistry*, vol. 281, no. 51, pp. 39728–39739, 2006.

[93] A. Postigo, M. C. Martin, M. P. Dodding, and M. Way, "Vaccinia-induced epidermal growth factor receptor-MEK signalling and the anti-apoptotic protein F1L synergize to suppress cell death during infection," *Cellular Microbiology*, vol. 11, no. 8, pp. 1208–1218, 2009.

[94] D. Zhai, E. Yu, C. Jin et al., "Vaccinia virus protein F1L is a caspase-9 inhibitor," *Journal of Biological Chemistry*, vol. 285, no. 8, pp. 5569–5580, 2010.

[95] M. Aoyagi, D. Zhai, C. Jin et al., "Vaccinia virus N1L protein resembles a B cell lymphoma-2 (Bcl-2) family protein," *Protein Science*, vol. 16, no. 1, pp. 118–124, 2007.

[96] C. Gubser, D. Bergamaschi, M. Hollinshead, X. Lu, F. J. van Kuppeveld, and G. L. Smith, "A new inhibitor of apoptosis from vaccinia virus and eukaryotes," *PLoS Pathogens*, vol. 3, no. 2, article e17, 2007.

[97] P. Zhang, J. O. Langland, B. L. Jacobs, and C. E. Samuel, "Protein kinase PKR-dependent activation of mitogen-activated protein kinases occurs through mitochondrial adapter IPS-1 and is antagonized by vaccinia virus E3L," *Journal of Virology*, vol. 83, no. 11, pp. 5718–5725, 2009.

[98] T. G. Senkevich, E. V. Koonin, and R. M. L. Buller, "A poxvirus protein with a RING zinc finger motif is of crucial importance for virulence," *Virology*, vol. 198, no. 2, pp. 118–128, 1994.

[99] T. G. Senkevich, E. J. Wolffe, and R. M. L. Buller, "Ectromelia virus RING finger protein is localized in virus factories and is required for virus replication in macrophages," *Journal of Virology*, vol. 69, no. 7, pp. 4103–4111, 1995.

[100] J. Huang, Q. Huang, X. Zhou et al., "The poxvirus p28 virulence factor is an E3 ubiquitin ligase," *Journal of Biological Chemistry*, vol. 279, no. 52, pp. 54110–54116, 2004.

[101] C. E. Samuel, "Antiviral actions of interferon interferon-regulated cellular proteins and their surprisingly selective antiviral activities," *Virology*, vol. 183, no. 1, pp. 1–11, 1991.

[102] N. L. Varich, I. V. Sychova, and N. V. Kaverin, "Transcription of both DNA strands of vaccinia virus genome in vivo," *Virology*, vol. 96, no. 2, pp. 412–420, 1979.

[103] P. Whitaker-Dowling and J. S. Youngner, "Characterization of a specific kinase inhibitory factor produced by vaccinia virus which inhibits the interferon-induced protein kinase," *Virology*, vol. 137, no. 1, pp. 171–181, 1984.

[104] J. C. Watson, H. W. Chang, and B. L. Jacobs, "Characterization of a vaccinia virus-encoded double-stranded RNA-binding protein that may be involved in inhibition of the double-stranded RNA-dependent protein kinase," *Virology*, vol. 185, no. 1, pp. 206–216, 1991.

[105] M. V. Davies, H. W. Chang, B. L. Jacobs, and R. J. Kaufman, "The E3L and K3L vaccinia virus gene products stimulate translation through inhibition of the double-stranded RNA-dependent protein kinase by different mechanisms," *Journal of Virology*, vol. 67, no. 3, pp. 1688–1692, 1993.

[106] E. Beattie, J. Tartaglia, and E. Paoletti, "Vaccinia virus-encoded eIF-2α homolog abrogates the antiviral effect of interferon," *Virology*, vol. 183, no. 1, pp. 419–422, 1991.

[107] P. R. Romano, F. Zhang, S. L. Tan et al., "Inhibition of double-stranded RNA-dependent protein kinase PKR by vaccinia virus E3: role of complex formation and the E3 N-terminal domain," *Molecular and Cellular Biology*, vol. 18, no. 12, pp. 7304–7316, 1998.

[108] T. A. Brandt and B. L. Jacobs, "Both carboxy- and amino-terminal domains of the vaccinia virus interferon resistance gene, E3L, are required for pathogenesis in a mouse model," *Journal of Virology*, vol. 75, no. 2, pp. 850–856, 2001.

[109] H. W. Chang, J. C. Watson, and B. L. Jacobs, "The E3L gene of vaccinia virus encodes an inhibitor of the interferon-induced, double-stranded RNA-dependent protein kinase," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 89, no. 11, pp. 4825–4829, 1992.

[110] C. Rivas, J. Gil, Z. Mělková, M. Esteban, and M. Díaz-Guerra, "Vaccinia virus E3L protein is an inhibitor of the interferon (IFN)- induced 2-5A synthetase enzyme," *Virology*, vol. 243, no. 2, pp. 406–414, 1998.

[111] S. N. Shchelkunov, A. V. Totmenin, P. F. Safronov et al., "Analysis of the monkeypox virus genome," *Virology*, vol. 297, no. 2, pp. 172–194, 2002.

[112] P. Bandi, N. E. Pagliaccetti, and M. D. Robek, "Inhibition of type III interferon activity by orthopoxvirus immunomodulatory proteins," *Journal of Interferon and Cytokine Research*, vol. 30, no. 3, pp. 123–133, 2010.

[113] B. A. Mann, J. H. Huang, P. Li et al., "Vaccinia virus blocks Stat1-dependent and Stat1-independent gene expression induced by type I and type II interferons," *Journal of Interferon and Cytokine Research*, vol. 28, no. 6, pp. 367–380, 2008.

[114] A. Alcami and G. L. Smith, "Vaccinia, cowpox, and camelpox viruses encode soluble gamma interferon receptors with novel broad species specificity," *Journal of Virology*, vol. 69, no. 8, pp. 4633–4639, 1995.

[115] J. A. Symons, A. Alcami, and G. L. Smith, "Vaccinia virus encodes a soluble type I interferon receptor of novel structure and broad species specificity," *Cell*, vol. 81, no. 4, pp. 551–560, 1995.

[116] S. N. Shchelkunov, "Immunomodulatory proteins of orthopoxviruses," *Molecular Biology*, vol. 37, no. 1, pp. 37–48, 2003.

[117] M. D. M. F. De Marco, A. Alejo, P. Hudson, I. K. Damon, and A. Alcami, "The highly virulent variola and monkeypox viruses express secreted inhibitors of type I interferon," *The FASEB Journal*, vol. 24, no. 5, pp. 1479–1488, 2010.

[118] K. J. Tracey and A. Cerami, "Tumor necrosis factor: a pleiotropic cytokine and therapeutic target," *Annual Review of Medicine*, vol. 45, pp. 491–503, 1994.

[119] G. J. Kotwal, "Microorganisms and their interaction with the immune system," *Journal of Leukocyte Biology*, vol. 62, no. 4, pp. 415–429, 1997.

[120] G. J. Kotwal, R. Blasco, C. G. Miller, S. Kuntz, S. Jayaraman, and S. N. Shchelkunov, "Strategies for immunomodulation and evasion by microbes: important consideration in the development of live vaccines," in *Symposium in Immunology VII*, M. M. Eibl and C. Huber, Eds., pp. 25–47, Springer, Berlin, Germany, 1998.

[121] A. Alcami and U. H. Koszinowski, "Viral mechanisms of immune evasion," *Immunology Today*, vol. 21, no. 9, pp. 447–455, 2000.

[122] G. J. Palumbo, D. J. Pickup, T. N. Fredrickson, L. J. McIntyre, and R. M. L. Buller, "Inhibition of an inflammatory response is mediated by a 38-kDa protein of cowpox virus," *Virology*, vol. 172, no. 1, pp. 262–273, 1989.

[123] G. J. Palumbo, W. C. Glasgow, and R. M. L. Buller, "Poxvirus-induced alteration of arachidonate metabolism," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 90, no. 5, pp. 2020–2024, 1993.

[124] A. Alcami and G. L. Smith, "A soluble receptor for interleukin-1β encoded by vaccinia virus: a novel mechanism of virus modulation of the host response to infection," *Cell*, vol. 71, no. 1, pp. 153–167, 1992.

[125] A. Alcamí and G. L. Smith, "A mechanism for the inhibition of fever by a virus," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 20, pp. 11029–11034, 1996.

[126] T. L. Born, L. A. Morrison, D. J. Esteban et al., "A poxvirus protein that binds to and inactivates IL-18, and inhibits NK cell response," *Journal of Immunology*, vol. 164, no. 6, pp. 3246–3254, 2000.

[127] S. N. Shchelkunov, V. M. Blinov, and L. S. Sandakhchiev, "Genes of variola and vaccinia viruses necessary to overcome the host protective mechanisms," *FEBS Letters*, vol. 319, no. 1-2, pp. 80–83, 1993.

[128] C. Upton, J. L. Macen, M. Schreiber, and G. McFadden, "Myxoma virus expresses a secreted protein with homology to the tumor necrosis factor receptor gene family that contributes to viral virulence," *Virology*, vol. 184, no. 1, pp. 370–382, 1991.

[129] C. A. Smith, F. Q. Hu, T. Davis Smith et al., "Cowpox virus genome encodes a second soluble homologue of cellular TNF receptors, distinct from CrmB, that binds TNF but not LTα," *Virology*, vol. 223, no. 1, pp. 132–147, 1996.

[130] V. N. Loparev, J. M. Parsons, J. C. Knight et al., "A third distinct tumor necrosis factor receptor of orthopoxviruses," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 7, pp. 3786–3791, 1998.

[131] A. Saraiva and A. Alcami, "CrmE, a novel soluble tumor necrosis factor receptor encoded by poxviruses," *Journal of Virology*, vol. 75, no. 1, pp. 226–233, 2001.

[132] I. P. Gileva, I. A. Ryazankin, Z. A. Maksyutov et al., "Comparative assessment of the properties of orthopoxviral soluble receptors for tumor necrosis factor," *Doklady Biochemistry and Biophysics*, vol. 390, pp. 160–164, 2003.

[133] I. P. Gileva, T. S. Nepomnyashchikh, D. V. Antonets et al., "Properties of the recombinant TNF-binding proteins from variola, monkeypox, and cowpox viruses are different," *Biochimica et Biophysica Acta*, vol. 1764, no. 11, pp. 1710–1718, 2006.

[134] A. Alejo, M. B. Ruiz-Argüello, Y. Ho, V. P. Smith, M. Saraiva, and A. Alcami, "A chemokine-binding domain in the tumor necrosis factor receptor from variola (smallpox) virus," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 15, pp. 5995–6000, 2006.

[135] D. V. Antonets, T. S. Nepomnyashchikh, and S. N. Shchelkunov, "SECRET domain of variola virus CrmB protein can be a member of poxviral type II chemokine-binding proteins family," *BMC Research Notes*, vol. 3, article e271, 2010.

[136] B. J. Rollins, "Chemokines," *Blood*, vol. 90, no. 3, pp. 909–928, 1997.

[137] A. H. Patel, D. F. Gaffney, J. H. Subak-Sharpe, and N. D. Stow, "DNA sequence of the gene encoding a major secreted protein of vaccinia virus, strain Lister," *Journal of General Virology*, vol. 71, no. 9, pp. 2013–2021, 1990.

[138] C. A. Smith, T. D. Smith, P. J. Smolak et al., "Poxvirus genomes encode a secreted, soluble protein that preferentially inhibits P chemokine activity yet lacks sequence homology to known chemokine receptors," *Virology*, vol. 236, no. 2, pp. 316–327, 1997.

[139] M. W. Bahar, J. C. Kenyon, M. M. Putz et al., "Structure and function of A41, a vaccinia virus chemokine binding protein," *PLoS Pathogens*, vol. 4, no. 1, article e5, 2008.

[140] M. B. Ruiz-Argüello, V. P. Smith, G. S. V. Campanella et al., "An ectromelia virus protein that interacts with chemokines through their glycosaminoglycan binding domain," *Journal of Virology*, vol. 82, no. 2, pp. 917–926, 2008.

[141] S. V. Seregin, I. N. Babkina, A. E. Nesterov, A. N. Sinyakov, and S. N. Shchelkunov, "Comparative studies of gamma-interferon receptor-like proteins of variola major and variola minor viruses," *FEBS Letters*, vol. 382, no. 1-2, pp. 79–83, 1996.

[142] C. Upton, K. Mossman, and G. McFadden, "Encoding of a homolog of the IFN-γ receptor by myxoma virus," *Science*, vol. 258, no. 5086, pp. 1369–1372, 1992.

[143] K. Mossman, C. Upton, R. M. L. Buller, and G. McFadden, "Species specificity of ectromelia virus and vaccinia virus interferon-γ binding proteins," *Virology*, vol. 208, no. 2, pp. 762–769, 1995.

[144] S. S. Marennikova and S. N. Shchelkunov, *Orthopoxviruses Pathogenic for Humans*, KMK Press, Moscow, Russia, 1998.

[145] G. J. Kotwal and B. Moss, "Vaccinia virus encodes a secretory polypeptide structurally related to complement control proteins," *Nature*, vol. 335, no. 6186, pp. 176–178, 1988.

[146] M. K. Liszewski and J. P. Atkinson, "Novel complement inhibitors," *Expert Opinion on Investigational Drugs*, vol. 7, no. 3, pp. 323–331, 1998.

[147] M. D. Kirkitadze, C. Henderson, N. C. Price et al., "Central modules of the vaccinia virus complement control protein are not in extensive contact," *Biochemical Journal*, vol. 344, no. 1, pp. 167–175, 1999.

[148] S. A. Smith, N. P. Mullin, J. Parkinson et al., "Conserved surface-exposed K/R-X-K/R motifs and net positive charge on poxvirus complement control proteins serve as putative heparin binding sites and contribute to inhibition of molecular interactions with human endothelial cells: a novel mechanism for evasion of host defense," *Journal of Virology*, vol. 74, no. 12, pp. 5659–5666, 2000.

[149] C. G. Miller, S. N. Shchelkunov, and G. J. Kotwal, "The cowpox virus-encoded homolog of the vaccinia virus complement control protein is an inflammation modulatory protein," *Virology*, vol. 229, no. 1, pp. 126–133, 1997.

[150] J. Howard, D. E. Justus, A. V. Totmenin, S. Shchelkunov, and G. J. Kotwal, "Molecular mimicry of the inflammation modulatory proteins (IMPS) of poxviruses: evasion of the inflammatory response to preserve viral habitat," *Journal of Leukocyte Biology*, vol. 64, no. 1, pp. 68–71, 1998.

[151] E. A. Uvarova and S. N. Shchelkunov, "Species-specific differences in the structure of orthopoxvirus complement-binding protein," *Virus Research*, vol. 81, no. 1-2, pp. 39–45, 2001.

[152] A. M. Rosengard, Y. Liu, Z. Nie, and R. Jimenez, "Variola virus immune evasion design: expression of a highly efficient

inhibitor of human complement," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 13, pp. 8808–8813, 2002.

[153] S. Jonjic, M. Babic, B. Polic, and A. Krmpotic, "Immune evasion of natural killer cells by viruses," *Current Opinion in Immunology*, vol. 20, no. 1, pp. 30–38, 2008.

[154] R. M. L. Buller and G. J. Palumbo, "Poxvirus pathogenesis," *Microbiological Reviews*, vol. 55, no. 1, pp. 80–122, 1991.

[155] J. A. Campbell, D. S. Trossman, W. M. Yokoyama, and L. N. Carayannopoulos, "Zoonotic orthopoxviruses encode a high-affinity antagonist of NKG2D," *Journal of Experimental Medicine*, vol. 204, no. 6, pp. 1311–1317, 2007.

[156] H. Okamura, H. Tsutsui, S. I. Kashiwamura, T. Yoshimoto, and K. Nakanishi, "Interleukin-18: a novel cytokine that augments both innate and acquired immunity," *Advances in Immunology*, vol. 70, pp. 281–312, 1998.

[157] M. K. Spriggs, "One step ahead of the game: viral immunomodulatory molecules," *Annual Review of Immunology*, vol. 14, pp. 101–130, 1996.

[158] W. E. Demkowicz and F. A. Ennis, "Vaccinia virus-specific $CD8^+$ cytotoxic T lymphocytes in humans," *Journal of Virology*, vol. 67, no. 3, pp. 1538–1544, 1993.

[159] K. E. Rehm, R. F. Connor, G. J. B. Jones, K. Yimbu, M. D. Mannie, and R. L. Roper, "Vaccinia virus decreases major histocompatibility complex (MHC) class II antigen presentation, T-cell priming, and peptide association with MHC class II," *Immunology*, vol. 128, no. 3, pp. 381–392, 2009.

[160] K. E. Rehm, R. F. Connor, G. J. B. Jones, K. Yimbu, and R. L. Roper, "Vaccinia virus A35R inhibits MHC class II antigen presentation," *Virology*, vol. 397, no. 1, pp. 176–186, 2010.

[161] K. E. Rehm and R. L. Roper, "Deletion of the A35 gene from modified vaccinia virus Ankara increases immunogenicity and isotype switching," *Vaccine*, vol. 29, no. 17, pp. 3276–3283, 2011.

[162] M. Byun, M. C. Verweij, D. J. Pickup, E. J. H. J. Wiertz, T. H. Hansen, and W. M. Yokoyama, "Two mechanistically distinct immune evasion proteins of cowpox virus combine to avoid antiviral CD8 T cells," *Cell Host and Microbe*, vol. 6, no. 5, pp. 422–432, 2009.

[163] A. Dasgupta, E. Hammarlund, M. K. Slifka, and K. Früh, "Cowpox virus evades CTL recognition and inhibits the intracellular transport of MHC class I molecules," *Journal of Immunology*, vol. 178, no. 3, pp. 1654–1661, 2007.

[164] P. M. Murphy, "Molecular mimicry and the generation of host defense protein diversity," *Cell*, vol. 72, no. 6, pp. 823–826, 1993.

[165] B. Beutler and C. van Huffel, "An evolutionary and functional approach to the TNF receptor/ligand family," *Annals of the New York Academy of Sciences*, vol. 730, pp. 118–133, 1994.

[166] S. N. Shchelkunov, R. F. Massung, and J. J. Esposito, "Comparison of the genome DNA sequences of Bangladesh-1975 and India-1967 variola viruses," *Virus Research*, vol. 36, no. 1, pp. 107–118, 1995.

[167] R. F. Massung, V. N. Loparev, J. C. Knight et al., "Terminal region sequence variations in variola virus DNA," *Virology*, vol. 221, no. 2, pp. 291–300, 1996.

[168] S. N. Shchelkunov, A. V. Totmenin, V. N. Loparev et al., "Alastrim smallpox variola minor virus genome DNA sequences," *Virology*, vol. 266, no. 2, pp. 361–386, 2000.

[169] S. N. Shchelkunov and A. V. Totmenin, "Two types of deletions in orthopoxvirus genomes," *Virus Genes*, vol. 9, no. 3, pp. 231–245, 1995.

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Review Article

Extracellular Vesicles and Their Convergence with Viral Pathways

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Extracellular vesicles (microvesicles), such as exosomes and shed microvesicles, contain a variety of molecules including proteins, lipids, and nucleic acids. Microvesicles appear mostly to originate from multivesicular bodies or to bud from the plasma membrane. Here, we review the convergence of microvesicle biogenesis and aspects of viral assembly and release pathways. Herpesviruses and retroviruses, amongst others, recruit several elements from the microvesicle biogenesis pathways for functional virus release. In addition, noninfectious pleiotropic virus-like vesicles can be released, containing viral and cellular components. We highlight the heterogeneity of microvesicle function during viral infection, addressing microvesicles that can either block or enhance infection, or cause immune dysregulation through bystander action in the immune system. Finally, endogenous retrovirus and retrotransposon elements deposited in our genomes millions of years ago can be released from cells within microvesicles, suggestive of a viral origin of the microvesicle system or perhaps of an evolutionary conserved system of virus-vesicle codependence. More research is needed to further elucidate the complex function of the various microvesicles produced during viral infection, possibly revealing new therapeutic intervention strategies.

1. An Introduction to Extracellular Vesicles

A wide variety of vesicles are actively released from living cells into the extracellular space with their contents reflecting the cellular composition and physiologic state (for review see [1–3]). Over the years, the different types of extracellular vesicles have been given a variety of names, including exosomes, shed microvesicles, ectosomes, microparticles, virosomes, virus-like particles, and oncosomes. The distinguishing features of each of the vesicle subtypes and the correct nomenclature are currently under intense study. Here, we will refer to them under the general term, microvesicles. Microvesicles carry RNA [mRNA, microRNA (miRNA), and noncoding sequences], cDNA and genomic sequences, and a large component of proteins and lipids (see reviews above, as well as [4, 5]). Upon release these microvesicles can move within the extracellular space and are either taken up by neighboring cells or degraded. They can also enter adjoining bodily fluids, such as the systemic circulation and travel to distant sites. In fact, they have been found in abundance in blood (serum and plasma), urine, breast milk, sweat, saliva, ascites fluid, and cerebral spinal fluid (CSF) [3–7]. At least two distinct release mechanisms for microvesicles have been described for two subtypes: (1) exosomes—derived from the multivesicular body (MVB) and (2) shed microvesicles—derived from the plasma membrane. Interestingly, both mechanisms have considerable overlap with virus release and biogenesis (summarized in Figure 1 and further discussed below).

Exosomes range from 30 to 100 nm in diameter and are generated by inward budding of the lumen of internal vesicular compartments derived from endosomes [8]. As vesicles accumulate within these endosome-derived compartments, they are referred to collectively as MVBs. These MVBs can either be targeted for degradation through the lysosomal pathway, or they can fuse with the plasma membrane releasing their interior vesicles into the extracellular space. The exact mechanism and kinetics of these fusion and release events are not fully elucidated and may vary among different cell types [9]. For example, depletion of Hrs (an ESCRT-0 component) led to a decrease in exosome secretion in dendritic cells that were stimulated to release with ovalbumin and a calcium ionophore [10]. Oligodendrocytes on the other hand seem to secrete exosomes by a mechanism that is ESCRT independent and ceramide dependent [11]. Exosome release by HeLa cells has been found to involve Rab27a/b [12], and p53 is reported to play a role in exosome release in a nonsmall cell lung cancer cell line [13]. Rab11 has also been shown to be involved in the release of exosomes from MVBs by acting in the tethering/docking of MVBs to the plasma membrane to promote homotypic fusion, in the presence of calcium [14]. In addition, TBC1D10A-C, a Rab35 inhibitor, led to intracellular accumulation of endosomal vesicles and impaired exosome secretion [15].

Shed microvesicles are released by outward budding directly from the plasma membrane and tend to be larger (>100 nm in diameter) and more heterogeneous in size [16, 17]. Moreover, this release process is likely controlled by localized cytoskeleton dynamics, with small cytoplasmic membrane-covered protrusions detaching and being released into the extracellular space [18] by an activated GTPase, ARF6 [19]. Interestingly, recent observations indicate that virus-independent budding from the plasma membrane can be mediated by endosome to plasma membrane relocation of TSG101, a prominent member of the ESCRT-I complex, frequently noted as an exosome marker [20]. This type of budding is topologically identical to both the inward budding of the limiting membrane of MVBs and viral assembly at the plasma membrane, in that the outer surface of the plasma membrane is on the outer surface of the microvesicle. In fact, certain tumor cells shed retroviral-like vesicles, which can be abundant because of increased transcription of endogenous retroviral sequences [17, 21], resulting from overall hypomethylation of the genome [22]. In general it seems that the clear distinctions between viruses and microvesicles based on composition and function are fading although they can be separated from vesicles released during the later stages of programmed cell death since these latter vesicles, referred to as apoptotic blebs [2], are even larger in size [23].

The role of microvesicles in intercellular communication is currently receiving much attention. Upon release from the donor cell, the microvesicles can either be taken up by neighboring cells or travel through bodily fluids for cargo delivery into recipient cells at distant sites. Although many details are missing, cellular uptake of some microvesicles appears to depend, at least in part, on specific ligand-receptor recognition [24], and can be mediated by direct fusion of the microvesicles with the plasma membrane or by endocytotic uptake of the microvesicles. For example, Quah et al. [25] have shown that bystander naïve B cells are rapidly activated by acquiring the antigen from activated B cells through microvesicle-mediated membrane transfer. In a similar way CD41 is transferred from platelets to endothelial and tumor cells, resulting in increased proadhesive properties of the recipient cells [26, 27]. Microvesicles also shuttle mRNA between cells and influence the physiological state of the recipient cell, as well as the cellular response to external stress stimuli [28]. In addition, miRNAs are transferred by exosomes [6, 29, 30]. For instance, miR-146a was shown to be transferred into recipient prostate cancer cells leading to the inhibition of their proliferation [31], and recently miRNAs which can modulate the immune response were detected in exosomes in breast milk [32]. Furthermore, retrotransposon sequences are particularly enriched in tumor microvesicles, and tumor-derived human endogenous retroviral (HERV) sequences can be transferred to normal human umbilical vein endothelial cells (HUVECs) via microvesicles resulting in a prolonged increase in HERV-K mRNA levels [17]. This suggests that tumor cells transfer these mobile genetic elements via microvesicles to neighboring normal cells thereby modulating their genotype and phenotype.

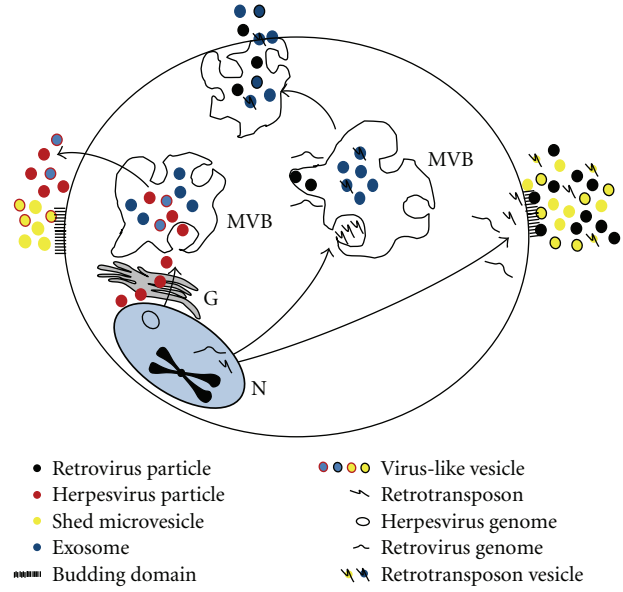

Figure 1: Convergence of microvesicle and virus biogenesis. Viruses share effectors of microvesicle production for their assembly and release. Exosomes produced in the MVB and shed microvesicles budding of the plasma membrane are indicated by blue and yellow dots, respectively. Extrachromosomal herpesvirus genomes are indicated by circles, retroviral genomes by sea-gull wings, and retrotransposons by the Y drawing. Herpesviruses, retroviruses, and retrotransposons sharing exosome or shed microvesicle pathways are indicated by red, black, or Y-containing dots, respectively. Chimeric virus-like vesicles are exosomes or shed microvesicles containing viral or retrotransposon elements and are indicated in dual color. N: nucleus, G: Golgi apparatus, MVB: multivesicular body.

2. Viruses and Microvesicles

Microvesicular shedding of cellular membrane components and the release of internal endosomal-derived exosomes are important for cellular communication and modulation of immune responses [9, 54–57] (Table 1). While release of microvesicles has been extensively investigated, recently the challenge has been to uncover the specific mechanisms that guide protein sorting and complexing into shed microvesicles and exosomes in various cell types. Cells have been reported to secrete highly specified microvesicles after infectious exposure or under various cell activation conditions [5, 54, 56, 58]. Through the packaging and transfer of functional proteins, mRNA/miRNA, and other cytosolic components, microvesicles have been found to be beneficial either to the host cell or to the infectious agent [37, 43]. Virus-infected cells proved useful in early studies to elucidate the role of microvesicular shedding in intercellular communication [55, 56]. Amongst the most extensively studied viruses with respect to microvesicles are herpes simplex virus (HSV), human immunodeficiency virus (HIV), and the tumorigenic herpes virus, Epstein-Barr virus (EBV). Each virus possesses unique properties that afford protection from immune attack. Here, we outline the important immune modulatory steps involved in virus-induced microvesicle sorting and release in these and other related viruses. Preservation of the virus depends on microvesicle release of infected cells. Microvesicles released by infected cells contain specific components of the cell and the virus, many of which facilitate the ability of virions to persist in a hostile antiviral immune environment [44, 55, 56, 58]. Depending on the virus type, and, in some cases, the stage in the viral cycle, intercellular processes are well orchestrated to produce specific cellular and immune outcomes [56]: (1) evading the host immune system, (2) invasion, (3) replication, and (4) persistence (summarized in part in Figure 2 and further discussed below).

2.1. Evading the Host Immune System. During primary viral infection, humoral and cell-mediated host immune responses such as production of neutralizing antibodies and cytotoxic T-cell attack on infected cells are employed to contribute to viral destruction. Early evasion strategies adopted by viruses interfere with complete elimination of the virus, allowing it to persist. During HSV-1 infection the release of microvesicles, formerly known as L-particles containing viral tegument proteins and glycoproteins, can prime surrounding cells for productive infection and reduce immune rejection [48–50]. Such virus-like vesicles lack both the viral capsid and DNA and are thereby incapable of producing a replication-infective cycle in the cells on their own [49–51]. However, some of the viral tegument proteins contained within them are immediate early transcription factors that can produce rapid transcriptional activation of later arriving intact virions [48, 52]. Another evasion strategy observed for HSV-1 is targeting of the MHCII molecule processing pathway by viral envelope glycoprotein B (gB) [37]. Antigen-presenting cells (APCs) routinely sort the MHCII surface receptor HLA-DR to MHCII compartments for processing. The primary role of this pathway is to present peptide antigens to the immune system in order to elicit or suppress T-(helper) cell responses that stimulate B-cell production of antigen-specific antibodies [37]. HSV-1 gB couples with HLA-DR, causing sorting through the exosome pathway as opposed to presentation on the cell surface. Complexing of gB-DR effectively hijacks the cellular antigen presenting machinery, preventing further peptide loading and, in addition, increasing microvesicle production [37, 53]. This final step releases additional gB-DR complexes into the host immune microenvironment, promoting resistance of viruses to immune attack, and in some cases producing bystander T-cell tolergenicity or anergy [37, 53]. In the case of HIV, microvesicle packaging and spread of the virus-encoded Nef protein impairs proper endocytosis of the immature MHCII/invariant chain, antibody class switching, and lysosomal degradation of viral peptides allowing HIV virus to evade immune recognition [37, 38]. EBV, human cytomegalovirus (CMV) and hepatitis C virus (HCV) have also found means to evade immune responses by exploiting microvesicles, as discussed below.

Table 1: Selective overview of viruses and vesicle function.

Virus	Immune status	Vesicle origin	Reference
HIV	Activating	CD8+ T cell	[33]
HIV	Activating	Megakaryocyte	[34, 35]
HIV	Activating	Dendritic cell	[36]
HIV	Evasion	Infected cell	[37–42]
CMV	Evasion	Infected cell	[43]
EBV	Evasion	Infected cell	[44–47]
HSV	Evasion	Infected cell	[37, 48–53]

2.2. Invasion and Replication within the Host Cell. Exosomes and shed microvesicles can both incorporate elements from the cell, as well as from the intruding virion [54]. Upon circulation of these microvesicles, they encounter and enter susceptible cells and can sensitize them to viral infection thus increasing systemic spread of the virus to naïve cells. In the case of the human CMV, microvesicles released by infected cells present the C-type lectin family molecule expressed on dendritic cells—used in capture and internalization of pathogens—in complex with the CMV glycoprotein B. This complex can be subsequently distributed to other cells by microvesicles, thereby increasing the susceptibility of these cells to CMV [59]. A similar mechanism is found in the case of HCV. In HCV-positive patients, the cellular membrane protein CD81 associates with one of the HCV envelope glycoproteins, E2. Extracellular release of the E2-CD81 complexes within microvesicles allows for increased virus-fusing ability and infectivity of previously naïve cells [60]. Microvesicles bearing the E2-CD81 complex and containing HCV RNA are of notable importance as they have been reported to be infectious even in the presence of neutralizing antibodies [60]. Interestingly, HCV has been shown to release three phenotypically distinct types of microvesicles having variable infectivity from high to low [60]. However, differential release of these microvesicles during HCV pathogenesis remains to be elucidated.

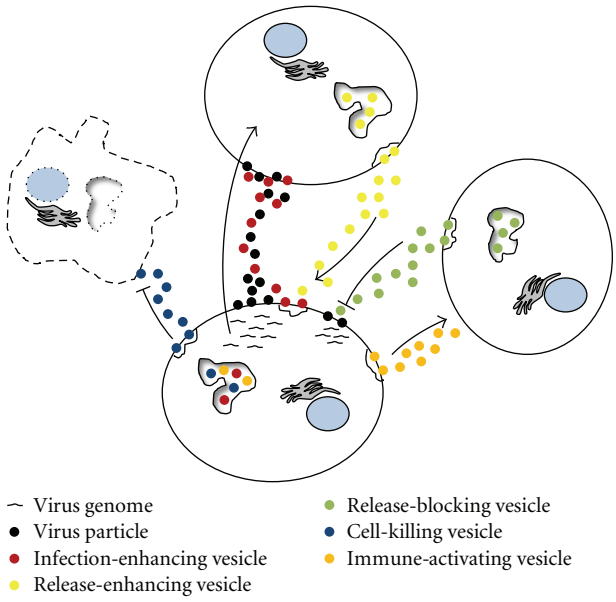

Figure 2: Heterogeneity of microvesicle function during virus infection. Microvesicles with diverse effects on virus spread are indicated by different colors. Microvesicles from infected cells can affect noninfected cells, enhancing infection, or killing incoming immune cells, or act to activate immune cells to viral antigens. Microvesicles from noninfected cells can either enhance or block virus release from the infected cell and modulate the immune response. Cell with dashed lines is undergoing cell death.

2.3. Microvesicles Contribute to Host Immunity against Viral Infection. Conversely, microvesicular release can contribute to viral attack by the host immune system. For example, in early invasion steps of CMV, CMV antigens are transferred from infected epithelial cells (ECs) via EC-derived microvesicles to APCs [43]. These APCs are not detected as infected cells but are rendered more susceptible to infection with subsequent encounters with the virus [43]. While this is a primary infectious viral invasion and replication strategy, inadvertently transferred APCs bearing CMV antigens in transplanted organs serve as markers to the host immune systems to target nonself tissue. Harboring of these susceptible APCs by the immune-compromised host and continued microvesicular shedding increases T-cell surveillance and influx into the grafted tissues, thereby exacerbating allograft rejection [43]. Microvesicles can also promote the innate immune response to viruses, for example, as observed for HIV whereby transfer of a particular antiviral cytidine deaminase via exosomes inhibits HIV replication [61]. In addition, virus-like vesicles can be used as a vaccination strategy, and recently chimeric virus-like vesicles were engineered using a mixture of coronavirus and influenza proteins functioning as a potential severe acute respiratory syndrome (SARS) virus vaccine [62].

2.4. Further Applications. Viruses can use various microvesicle transport mechanisms as a survival strategy, while in other cases the host immune system can utilize microvesicles for cell signaling and host protection. Microvesicles can directly activate or suppress cellular responses, induce or facilitate infection, and transfer material to improve or hinder host immune recognition [9]. These same strategies can be exploited in the development of virus-based therapies. Oncolytic viruses armed with therapeutic genes are currently being evaluated for safety and efficacy for cancer therapy [63–65]. It would be of interest to determine whether microvesicles can alter the efficacy of oncolytic viruses, and other types of viral gene delivery vectors. Recent work shows that microvesicles can be loaded with adenoassociated viral (AAVs) vectors for more efficient gene delivery [66], opening a new window into the microvesicle therapeutics field.

3. EBV and Microvesicles

Several human pathogenic viruses are known for their ability to lie dormant in the host immune system, of which HSV and EBV are perhaps the best known examples. In the case of HSV this is due to the ability of the virus to enter a latent state in the nucleus of sensory neurons during which it expresses no viral antigens and does not disturb the physiology of the neurons. In latency a single transcript is generated which encodes a precursor for four distinct HSV, miRNAs which act to suppress virus replication [67]. For human herpesvirus 4 (HHV4), better known as EBV, this is largely due to incomplete eradication of the virus after early primary infection.

Gamma herpesviruses, including EBV, have developed a variety of strategies to exploit host-cell regulatory pathways that lead to a permanent infection of their host. When these pathways are deregulated, what is usually an undamaging herpes infection can predispose to disease-including encephalitis, autoimmunity, and cancer [68]. It was recently demonstrated that EBV exploits the endosomal-exosomal pathway by balancing intracellular signaling in infected B cells [69] and controlling epigenetic changes in uninfected neighboring cells via microvesicles [30]. Enveloped viruses of the herpes virus family, such as human CMV (HCMV/HHV5) and EBV, depend on the interaction with cellular endosomal membrane systems for replication [70]. Interestingly, mature HHV-6 virions are released together with internal vesicles through MVBs by the cellular endosomal-exosomal pathway [71]. Thus, many herpesviruses generally seem to exploit endosomal pathways and microvesicles for virus production, release, and immune evasion. However, the finding that viruses such as EBV modulate host-cellular pathways that are not directly involved in virus production needs further investigation.

Being the first human tumor virus identified, EBV is in many aspects an extraordinarily benign pathogen and is best known as the causative agent of "kissing disease" or infectious mononucleosis. It is estimated that over 90% of the world population is persistently infected with EBV. The EBV life cycle begins by exchange through saliva and EBV virions that seem to preferentially infect naïve resting B cells in secondary lymphoid organs, such as the tonsils. Occasionally isolated epithelial cells also become infected and presumably sustain lytic replication [72], which is required for viral shedding into the saliva for transmission to new hosts [73].

To reach its near universal prevalence without harming the host, EBV and related persistent herpesviruses have evolved complex strategies encouraging immune recognition in proliferative (potentially oncogenic) stages of its life cycle, while elegantly avoiding the immune recognition at other stages by "going into hiding" [74]. Upon initial infection at the mantle zone of germinal centers (GCs), the newly infected naïve B cells undergo multiple differentiation stages and tight interactions with surrounding stroma and T cells [75]. Interestingly, EBV facilitates these essential interactions for the maturation of B cells, for instance, by upregulation of crucial GC reaction-associated proteins, such as GP183 [76]. This integral part of the EBV life cycle (i.e., mimicking a GC-type reaction) requires tight growth regulation in a specific EBV latency gene expression program (Latency III) and promotes rapid growth and proliferation of these infected cells through NFκB activation. This strategy in expanding the infected pool of B cells without the need for lytic replication may be advantageous under normal conditions but raises the chances of turning-on malignant growth if the viral latency programs are not properly controlled. Indeed, if these cells do not progress further into memory cells by shutting down this growth program, they can remain in the proliferative phase and give rise to EBV-positive lymphomas which can kill the host, thus, restricting further viral propagation and spread [77]. In addition, EBV infection at this stage may also predispose to autoimmunity as inappropriate survival signals may interfere with negative selection of self-reactive B cells. Of note, immune-suppressed individuals are at increased risk for developing EBV-driven lymphomas, reflecting the importance of a lifelong potent anti-EBV T-cell response [78]. The ability of EBV to persist despite such vigorous T-cell responses indicates that EBV can escape from the adaptive immune system and may do so in part by exploiting the endosomal-exosomal pathway through the secretion of T-cell inhibitory exosomes [44–46]. When secreted by EBV-positive tumors, these exosomes carry immune-evasive proteins including the viral protein LMP1 [79] and high amounts of galectin 9 that cause massive apoptosis of EBV-specific CD4+ T-cells via specific interaction with T-cell immunoglobulin mucin-3 (Tim-3), which can negatively regulate Th1 T cell and macrophage activation. The inhibition of anti-EBV immune responses is believed to promote the progression of EBV-positive malignancies, such as Hodgkin's disease (HD) [46] and nasopharyngeal carcinoma (NPC) [80].

Vallhov et al. [81] studied the interaction between exosomes secreted by EBV-driven lymphoblastoid cell lines (LCLs) and peripheral blood B cells proliferating *in vitro*. LCLs are 95% latent, but a small proportion of cells is in a lytic stage. Exosome-cell interactions could be inhibited by specific antibodies against gp350 the major envelope protein of EBV or CD21 on B cells, indicating an interaction between CD21 on B cells and the gp350 on exosomes [81]. These specific exosome-cell interactions may be exploited for exosome-based anticancer therapies, for example, in delivering the CD154 protein to leukemic B blast cells rendering them immunogenic to T cells [82]. In addition to proteins, it is now clear that microvesicles from many cell types carry and transport functional RNA molecules. EBV was the first virus discovered to encode its own small regulatory miRNAs [83]. EBV encodes a staggering 44 viral miRNA species, derived from two major gene clusters on the viral genome, which have an important role in EBV persistence [84]. Next generation sequencing indicates that these EBV-encoded miRNAs make up a large fraction (20–25%) of the total cellular miRNA in EBV-infected cells, encompassing 300+ different miRNA species [85]. Similar results were found in the miRNA profile of exosomes from EBV-driven LCL cells (Pegtel et al., unpublished results). This is consistent with the idea that viral miRNAs manipulate gene regulation in host cellular pathways and also exploit the exosomal miRNA communication pathways.

Indeed, the discovery of EBV-encoded regulatory miRNAs (EBV-miRNAs) residing within the lumen of exosomes indicated a novel mechanism by which exosomes can exert inhibitory effects, namely, by translational repression of target genes in noninfected recipient cells via exosomal EBV miRNAs [30]. Earlier studies in mice had suggested that intact exosomes from EBV-infected cells had strong physiological effects *in vivo*, consistent with the idea that the luminal content of exosomes is biologically significant, apart from the proteins and lipids that make up their surface [86]. Subsequent studies demonstrated that EBV-infected cancer ECs also secrete EBV-miRNAs, presumably within exosomes [87]. Due to the lack of an accurate *in vivo* model for human EBV infection it is difficult to investigate the mechanism controlling release of EBV-miRNAs through exosomes and to determine whether this contributes to viral persistence in healthy infected individuals. However, EBV-encoded miRNAs are transported from infected B cells to noninfected (EBV-DNA negative) T cells and monocytes, supporting the idea of horizontal miRNA transfer in humans. Thus, viral miRNAs in exosomes may contribute to sustain persistent virus infection by delivery of such miRNAs into noninfected responding T cells leading to their inactivation (anergy) [45] or destruction [44]. This is consistent with recent data suggesting that exosomes efficiently transport miRNAs through the immunological synapse during interactions of T cells with APCs [47], similar to what is known concerning antigen exchange [88]. Studies are underway to establish whether EBV exploits these specialized intercellular contacts for efficient posttranscriptional control in neighboring responding immune cells as a possible mechanism for immune escape.

4. HIV and Microvesicles

HIV [56, 89–91] has been a discussion topic in the microvesicle field for many years. Not only has it been hypothesized that HIV itself may have microvesicle features, but microvesicles also have been described to have immune modulatory functions on HIV-infected cells and to expand the infectivity of HIV.

In 2003 Gould et al. [92] postulated that HIV—an enveloped retrovirus—hijacks the microvesicle system to benefit its own assembly and subsequent exit. Interestingly,

inhibitors were identified that blocked the budding of both shed microvesicles and HIV particles [93]. In addition, peptides were identified that prevented interactions of HIV Nef protein—a key protein in the HIV life cycle—with mortalin, a cellular heat shock protein, and resulted in inhibition of the release of HIV and Nef-containing microvesicles [94]. Careful analysis, however, has indicated that although HIV exploits certain proteins that also play a role in exosome formation via the MVB [95], HIV assembly does not necessarily use the same logistics system as do exosomes. Importantly, it has been established that HIV budding occurs mostly at the plasma membrane and not from within the MVB [96–99]. Interestingly, HIV recruits members of the MVB ESCRT complex for proper HIV budding from the plasma membrane [98–102]. While in CD4+ T cells HIV release appears to be independent of exosomes [103], in monocyte-derived macrophages HIV can bud into endosomes [102, 104]. However, several studies highlight that HIV-1 budding also in macrophages occurs primary at the plasma membrane [105–107]. Thus, the controversy about the site of productive virus assembly in macrophages mostly favors the plasma membrane. HIV release in dendritic cells may be triggered by signals similar to those for exosome release [102, 108, 109], and secretion of HIV from endocytic compartments in dendritic cells can result in HIV release upon interaction with T cells [110, 111]. However, these endocytic compartments were also described to be connected with the extracellular space [112, 113] and suggested to be invaginated domains distinct from classical endocytic vesicles [114]. Moreover, microvesicle release from T cells treated with ceramide inhibitors was not affected by such treatment [111], as previously reported for HIV-1 [115]. However, both viruses and microvesicles produced from ceramide-deficient cells failed to be captured by mature dendritic cells [111]. Therefore, more research is warranted on the specific sites of HIV assembly in particular cell types, and to what extent the endosomal compartments play a role in the HIV life cycle, as well as the possible convergence of HIV and shed microvesicle pathways.

It seems likely that HIV has simply adapted to use certain host factors for different exit modalities, and that these may vary among different types of cells, as well as under different conditions. It will be of continuing interest to further study the retroviral family, including the endogenous retroviruses, in order to determine whether the microvesicle cargo systems are perhaps a remnant of previous retroviral infections that happened earlier in evolution—and elements of which are now used in an opportunistic setting by retroviruses, such as HIV [56, 89–91, 102]. This overlap in pathways and the consequence of using overlapping machinery for release can result in phenotypic similarities between microvesicles and retroviruses and potentially interfere with anti-HIV strategies. For instance, HIV released from T cells has similar glycome properties as T-cell microvesicles, arguing for a common origin and indicating phenotypic similarity [116]. More research in the convergence of microvesicle and HIV pathways may improve our understanding of these processes and propel the development of new antiviral drugs directed against HIV.

The role of microvesicles during HIV infection has not yet been extensively studied, but they appear to be involved in both HIV infectivity enhancement and resistance depending on the cells of origin. Microvesicles derived from HIV-infected cells have been reported to contain HIV CCR5 coreceptors, allowing for enhanced HIV infection of other cells [34]. Moreover, microvesicles from megakaryocytes and platelets contain CXCR4 and upon transference confer susceptibility to cells normally resistant to HIV infection [35, 117]. In addition, during HIV replication the HIV Nef protein can alter the exosomal pathway by increasing the number of intracellular vesicles and MVBs [118–121]. HIV Nef-induced microvesicle release from infected and noninfected cells [39, 40] can induce apoptosis in CD4+ T cells [41] and convey resistance to HIV infection [61]. The transfer of Nef or other viral components through microvesicles may represent an important mechanism for immune evasion by viruses. In addition, exosomes can contain APOBEC3G, a cytidine deaminase that is part of the cellular antiviral system against retroviruses, which upon transference to recipient cells via exosomes can inhibit HIV replication [61]. While CD45, CD86, and MHC Class II molecules have been found in microvesicles from HIV-infected cells [42], possibly serving to silence the immune response, microvesicles derived from CD8+ T cells can act to suppress HIV replication [33]. Moreover, exosomes in association with HIV derived from dendritic cells significantly enhance HIV infection of CD4+ T cells [36]. In conclusion, microvesicles from HIV-infected cells as well as from noninfected cells play an important role in HIV replication and dissemination. Therefore, interference with microvesicle-mediated signaling could possibly be harnessed to halt HIV infection.

5. Retrotrasposon Elements and Microvesicles

Retrotransposon elements such as LINE, Alu, and human endogenous retroviruses (HERVs) make up about 45% of the human genome and have played an important role in genome evolution [122]. These viral-like elements infected germ cells in the human genome millions of years ago and then became a stable part of the inherited genetic material. Although most LINE elements are inactive, a number of active ones remain and are able to "jump" to new locations in the genome, contributing to genomic instability [123]. These events can have important effects on our genome, for example, by inactivating genes, altering gene expression and facilitating random insertion of new cDNA copies in the genome, as in integration of pseudogenes [124]. Many tumor cells also release retroviral-like microvesicles that contain active retrotransposon sequences, such as HERV-K [125].

Recently, tumor-derived microvesicles have been shown to be enriched in retrotransposon elements such as LINE1, Alu, and HERV-K [17]. Furthermore, HERV-K was transferred through microvesicles to normal HUVECs, which then showed an increase in HERV-K levels 12 hours following exposure to tumor microvesicles. In addition, the mouse retroviral RNA VL30 is packaged in retrovirus vectors by mouse packaging cell lines and transferred to human

cells infected with those vectors [126]. The mouse VL30 has several stop codons in the regions encoding for genes such as *gag*, *pol* and *env*, thereby inhibiting its ability to encode functional proteins [126]. However, transfer of the VL30 mRNA together with tissue factor (TF) to human melanoma cells served to induce their metastatic potential. This change in phenotype apparently occurs through formation of a complex with the protein-associated splicing factor (PSF) protein which represses transcription of an insulin-like growth factor-1 (IGF-1) inducible gene, with dissociation of this complex allowing transcription to proceed [126]. Three of the 11 human genes affected by VL30 mRNA were oncogenic, suggesting that the transfer of retroviral RNA sequences can have catastrophic effects on recipient cells. Song et al. [126] have identified human retrotransposon sequences that are >90% identical to the mouse VL-30 suggesting human VL-30 transferred through microvesicles could have similar effects on transcription [126].

Long interspersed elements (LINEs)—most notably L1—comprise about 17% of the human genome. Several studies indicate that a subset of L1 elements is still actively expanding in the number of sequences within the human genome by retrotransposition. This active subpopulation, termed transcriptionally active (Ta), is approximately 2 million years old, and it has high levels of insertional polymorphism in the human population [127, 128]. Some of these new insertions may be intolerable and lethal and therefore eliminated; others may result in phenotypically tolerable disease, such as in Coffin-Lowry Syndrome and choroideremia [129–131], while still others have been associated with the induction of cancer, for example, lung cancer [132]. The high level of polymorphism of L1 elements indicates that they continue to have profound effects on the human genome, and recent evidence suggests that microvesicles may be a potential route of delivery for these elements [17]. This microvesicle-mediated Trojan Horse-like [92] transferance of transposons could perhaps allow for a stealthy dissemination of retrotransposons, especially in a tumor setting, avoiding immune-recognition, and achieving "long distance" delivery.

HERVs also entered the human genome millions of years ago and comprise about 8% of the human genome. They consist of *gag*, *pol,* and *env* sequences, flanked by two long terminal repeats [133]. Most of these sequences are now silent because of acquired mutations and deletions over the course of evolution, but HERV-K113 can produce intact, albeit noninfectious, retroviral particles [134]. Some of these sequences are still transcriptionally active and are associated with diseases, such as lymphoma and breast cancer [21, 135]. In cancer, hypomethylation of the genome seems to predominantly affect retrotransposon sequences (perhaps because they are highly abundant in the human genome), allowing increased transcription, especially in the case of the most recent entrants, which also happen to be the elements with the most intact coding potential [136]. Indeed retroviral-like microvesicles have been found in cancer patients, notably those with lymphomas [21], breast cancer [137], and teratomas [138]. As expected, these patients also had high levels of reverse transcriptase, and viral gag and env proteins and RNA in the tumor cells and retrovirus-like microvesicles released from them into the circulation [21]. Tumor microvesicles from cultured tumor cells also have been shown to be enriched in retrotransposon RNA, DNA, and reverse transcriptase, suggesting that a subpopulation of these microvesicles may indeed be of retroviral origin [19].

6. Concluding Remarks

In summary, this review deals with how extracellular vesicles—such as exosomes and shed microvesicles—share pathways with the assembly and release of retrotransposon elements and viruses. In Figure 1 we summarize how herpesviruses such as EBV and HSV, originate from the nucleus and can merge with microvesicle pathways. Several proteins used for exosome production are used by herpesviruses for functional release. Also, the convergence of these pathways may explain the observations of virus-like particles, which can be exosomes or shed microvesicles containing viral proteins or nucleic acids. Similar observations have been made for retroviruses and retrotransposon elements with circulating microvesicles containing retrotransposon RNA found in some cancer patients. It remains to be investigated to what extent exosomes and shed microvesicles are remnants of previous retroviral colonization. In this review we note the observations of retroviral as well as retrotransposon elements in microvesicles, perhaps allowing further dissemination of such nucleic acid sequences. The use of microvesicle pathway elements by viruses such as HIV may be suggestive of an intricate coevolution of different endogenous and exogenous (retro)virus subtypes. Viruses not only use microvesicle pathways for their own assembly and release but are also capable of exploiting the highly complex microvesicle communication system in an intercellular setting as simplified in Figure 2. During viral infection microvesicles can have various effects on different types of cells, either limiting viral infection or enhancing it. Thus, a picture is emerging that viruses and microvesicles are codependent pleiotropic entities. More research is needed into the differential functions of different subtypes of microvesicles and their cross-talk in relation to the immune response and outcome of viral infection.

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References

[1] E. Cocucci, G. Racchetti, and J. Meldolesi, "Shedding microvesicles: artefacts no more," *Trends in Cell Biology*, vol. 19, no. 2, pp. 43–51, 2009.

[2] S. Mathivanan, H. Ji, and R. J. Simpson, "Exosomes: extracellular organelles important in intercellular communication," *Journal of Proteomics*, vol. 73, no. 10, pp. 1907–1920, 2010.

[3] M. Simons and G. Raposo, "Exosomes—vesicular carriers for intercellular communication," *Current Opinion in Cell Biology*, vol. 21, no. 4, pp. 575–581, 2009.

[4] K. E. van der Vos, L. Balaj, J. Skog, and X. O. Breakefield, "Brain tumor microvesicles: insights into intercellular communication in the nervous system," *Cellular and Molecular Neurobiology*, vol. 31, pp. 949–959, 2011.

[5] S. Pant, H. Hilton, and M. E. Burczynski, "The multifaceted exosome: biogenesis, role in normal and aberrant cellular function, and frontiers for pharmacological and biomarker opportunities," *Biochemical Pharmacology*, vol. 83, no. 11, pp. 1484–1494, 2012.

[6] J. Skog, T. Würdinger, S. van Rijn et al., "Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers," *Nature Cell Biology*, vol. 10, no. 12, pp. 1470–1476, 2008.

[7] C. Lässer, V. Seyed Alikhani, K. Ekström et al., "Human saliva, plasma and breast milk exosomes contain RNA: uptake by macrophages," *Journal of Translational Medicine*, vol. 9, article 9, 2011.

[8] C. Théry, A. Regnault, J. Garin et al., "Molecular characterization of dendritic cell-derived exosomes: selective accumulation of the heat shock protein hsc73," *Journal of Cell Biology*, vol. 147, no. 3, pp. 599–610, 1999.

[9] C. Théry, M. Ostrowski, and E. Segura, "Membrane vesicles as conveyors of immune responses," *Nature Reviews Immunology*, vol. 9, no. 8, pp. 581–593, 2009.

[10] K. Tamai, N. Tanaka, T. Nakano et al., "Exosome secretion of dendritic cells is regulated by Hrs, an ESCRT-0 protein," *Biochemical and Biophysical Research Communications*, vol. 399, no. 3, pp. 384–390, 2010.

[11] K. Trajkovic, C. Hsu, S. Chiantia et al., "Ceramide triggers budding of exosome vesicles into multivesicular endosomes," *Science*, vol. 319, no. 5867, pp. 1244–1247, 2008.

[12] M. Ostrowski, N. B. Carmo, S. Krumeich et al., "Rab27a and Rab27b control different steps of the exosome secretion pathway," *Nature Cell Biology*, vol. 12, no. 1, pp. 19–3013, 2010.

[13] X. Yu, S. L. Harris, and A. J. Levine, "The regulation of exosome secretion: a novel function of the p53 protein," *Cancer Research*, vol. 66, no. 9, pp. 4795–4801, 2006.

[14] A. Savina, C. M. Fader, M. T. Damiani, and M. I. Colombo, "Rab11 promotes docking and fusion of multivesicular bodies in a calcium-dependent manner," *Traffic*, vol. 6, no. 2, pp. 131–143, 2005.

[15] C. Hsu, Y. Morohashi, S. I. Yoshimura et al., "Regulation of exosome secretion by Rab35 and its GTPase-activating proteins TBC1D10A-C," *Journal of Cell Biology*, vol. 189, no. 2, pp. 223–232, 2010.

[16] K. Al-Nedawi, B. Meehan, J. Micallef et al., "Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells," *Nature Cell Biology*, vol. 10, no. 5, pp. 619–624, 2008.

[17] L. Balaj, R. Lessard, L. Dai et al., "Tumour microvesicles contain retrotransposon elements and amplified oncogene sequences," *Nature Communications*, vol. 2, article 180, 2011.

[18] K. Schara, V. Janša, V. Šuštar et al., "Mechanisms for the formation of membranous nanostructures in cell-to-cell communication," *Cellular and Molecular Biology Letters*, vol. 14, no. 4, pp. 636–656, 2009.

[19] V. Muralidharan-Chari, J. Clancy, C. Plou et al., "ARF6-regulated shedding of tumor cell-derived plasma membrane microvesicles," *Current Biology*, vol. 19, no. 22, pp. 1875–1885, 2009.

[20] J. F. Nabhan, R. Hu, R. S. Oh, S. N. Cohen, and Q. Lu, "Formation and release of arrestin domain-containing protein 1-mediated microvesicles (ARMMs) at plasma membrane by recruitment of TSG101 protein," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 109, no. 11, pp. 4146–4151, 2012.

[21] R. Contreras-Galindo, M. H. Kaplan, P. Leissner et al., "Human endogenous retrovirus K (HML-2) elements in the plasma of people with lymphoma and breast cancer," *Journal of Virology*, vol. 82, no. 19, pp. 9329–9336, 2008.

[22] A. Daskalos, G. Nikolaidis, G. Xinarianos et al., "Hypomethylation of retrotransposable elements correlates with genomic instability in non-small cell lung cancer," *International Journal of Cancer*, vol. 124, no. 1, pp. 81–87, 2009.

[23] M. Hristov, W. Erl, S. Linder, and P. C. Weber, "Apoptotic bodies from endothelial cells enhance the number and initiate the differentiation of human endothelial progenitor cells in vitro," *Blood*, vol. 104, no. 9, pp. 2761–2766, 2004.

[24] W. Lösche, T. Scholz, U. Temmler, V. Oberle, and R. A. Claus, "Platelet-derived microvesicles transfer tissue factor to monocytes but not to neutrophils," *Platelets*, vol. 15, no. 2, pp. 109–115, 2004.

[25] B. J. C. Quah, V. P. Barlow, V. McPhun, K. I. Matthaei, M. D. Hulett, and C. R. Parish, "Bystander B cells rapidly acquire antigen receptors from activated B cells by membrane transfer," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 11, pp. 4259–4264, 2008.

[26] O. P. Barry, D. Praticò, R. C. Savani, and G. A. FitzGerald, "Modulation of monocyte-endothelial cell interactions by platelet microparticles," *Journal of Clinical Investigation*, vol. 102, no. 1, pp. 136–144, 1998.

[27] A. Janowska-Wieczorek, M. Majka, J. Kijowski et al., "Platelet-derived microparticles bind to hematopoietic stem/progenitor cells and enhance their engraftment," *Blood*, vol. 98, no. 10, pp. 3143–3149, 2001.

[28] M. Eldh, K. Ekström, H. Valadi et al., "Exosomes communicate protective messages during oxidative stress; possible role of exosomal shuttle RNA," *PloS One*, vol. 5, no. 12, Article ID e15353, 2010.

[29] H. Valadi, K. Ekström, A. Bossios, M. Sjöstrand, J. J. Lee, and J. O. Lötvall, "Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells," *Nature Cell Biology*, vol. 9, no. 6, pp. 654–659, 2007.

[30] D. M. Pegtel, K. Cosmopoulos, D. A. Thorley-Lawson et al., "Functional delivery of viral miRNAs via exosomes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 14, pp. 6328–6333, 2010.

[31] N. Kosaka and T. Ochiya, "Unraveling the mystery of cancer by secretory microRNA: horizontal microRNA transfer between living cells," *Frontiers in Genetics*, vol. 2, p. 97, 2011.

[32] Q. Zhou, M. Li, X. Wang et al., "Immune-related microRNAs are abundant in breast milk exosomes," *International Journal of Biological Sciences*, vol. 8, no. 1, pp. 118–123, 2011.

[33] A. Tumne, V. S. Prasad, Y. Chen et al., "Noncytotoxic suppression of human immunodeficiency virus type 1 transcription by exosomes secreted from $CD8^+$ T cells," *Journal of Virology*, vol. 83, no. 9, pp. 4354–4364, 2009.

[34] M. Mack, A. Kleinschmidt, H. Brühl et al., "Transfer of the chemokine receptor CCR5 between cells by membrane-derived microparticles: a mechanism for cellular human immunodeficiency virus 1 infection," *Nature Medicine*, vol. 6, no. 7, pp. 769–775, 2000.

[35] T. Rozmyslowicz, M. Majka, J. Kijowski et al., "Platelet- and megakaryocyte-derived microparticles transfer CXCR4 receptor to CXCR4-null cells and make them susceptible to infection by X4-HIV," *AIDS*, vol. 17, no. 1, pp. 33–42, 2003.

[36] R. D. Wiley and S. Gummuluru, "Immature dendritic cell-derived exosomes can mediate HIV-1 trans infection," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 3, pp. 738–743, 2006.

[37] S. Temme, A. M. Eis-Hübinger, A. D. McLellan, and N. Koch, "The herpes simplex virus-1 encoded glycoprotein B diverts HLA-DR into the exosome pathway," *The Journal of Immunology*, vol. 184, no. 1, pp. 236–243, 2010.

[38] W. Xu, P. A. Santini, J. S. Sullivan et al., "HIV-1 evades virus-specific IgG2 and IgA responses by targeting systemic and intestinal B cells via long-range intercellular conduits," *Nature Immunology*, vol. 10, no. 9, pp. 1008–1017, 2009.

[39] C. Muratori, L. E. Cavallin, K. Krätzel et al., "Massive secretion by T cells is caused by HIV Nef in infected cells and by Nef transfer to bystander cells," *Cell Host and Microbe*, vol. 6, no. 3, pp. 218–230, 2009.

[40] S. A. Ali, M. B. Huang, P. E. Campbell et al., "Genetic characterization of HIV type 1 nef-induced vesicle secretion," *AIDS Research and Human Retroviruses*, vol. 26, no. 2, pp. 173–192, 2010.

[41] M. Lenassi, G. Cagney, M. Liao et al., "HIV Nef is secreted in exosomes and triggers apoptosis in bystander $CD4^+$ T cells," *Traffic*, vol. 11, no. 1, pp. 110–122, 2010.

[42] M. T. Esser, D. R. Graham, L. V. Coren et al., "Differential incorporation of CD45, CD80 (B7-1), CD86 (B7-2), and major histocompatibility complex class I and II molecules into human immunodeficiency virus type 1 virions and microvesicles: implications for viral pathogenesis and immune regulation," *Journal of Virology*, vol. 75, no. 13, pp. 6173–6182, 2001.

[43] J. D. Walker, C. L. Maier, and J. S. Pober, "Cytomegalovirus-infected human endothelial cells can stimulate allogeneic $CD4^+$ memory T cells by releasing antigenic exosomes," *The Journal of Immunology*, vol. 182, no. 3, pp. 1548–1559, 2009.

[44] J. Klibi, T. Niki, A. Riedel et al., "Blood diffusion and Th1-suppressive effects of galectin-9-containing exosomes released by Epstein-Barr virus-infected nasopharyngeal carcinoma cells," *Blood*, vol. 113, no. 9, pp. 1957–1966, 2009.

[45] J. Flanagan, J. Middeldorp, and T. Sculley, "Localization of the Epstein-Barr virus protein LMP 1 to exosomes," *Journal of General Virology*, vol. 84, no. 7, pp. 1871–1879, 2003.

[46] M. K. Gandhi, G. Moll, C. Smith et al., "Galectin-1 mediated suppression of Epstein-Barr virus-specific T-cell immunity in classic Hodgkin lymphoma," *Blood*, vol. 110, no. 4, pp. 1326–1329, 2007.

[47] M. Mittelbrunn, C. Gutiérrez-Vázquez, C. Villarroya-Beltri et al., "Unidirectional transfer of microRNA-loaded exosomes from T cells to antigen-presenting cells," *Nature Communications*, vol. 2, no. 1, article 282, 2011.

[48] D. J. Dargan and J. H. Subak-Sharpe, "The effect of herpes simplex virus type 1 L-particles on virus entry, replication, and the infectivity of naked herpesvirus DNA," *Virology*, vol. 239, no. 2, pp. 378–388, 1997.

[49] S. Loret, G. Guay, and R. Lippé, "Comprehensive characterization of extracellular herpes simplex virus type 1 virions," *Journal of Virology*, vol. 82, no. 17, pp. 8605–8618, 2008.

[50] J. McLauchlan, C. Addison, M. C. Craigie, and F. J. Rixon, "Noninfectious L-particles supply functions which can facilitate infection by HSV-1," *Virology*, vol. 190, no. 2, pp. 682–688, 1992.

[51] F. J. Rixon, C. Addison, and J. Mclauchlan, "Assembly of enveloped tegument structures (L particles) can occur independently of virion maturation in herpes simplex virus type 1-infected cells," *Journal of General Virology*, vol. 73, no. 2, pp. 277–284, 1992.

[52] B. J. Kelly, C. Fraefel, A. L. Cunningham, and R. J. Diefenbach, "Functional roles of the tegument proteins of herpes simplex virus type 1," *Virus Research*, vol. 145, no. 2, pp. 173–186, 2009.

[53] J. Neumann, A. M. Eis-Hübinger, and N. Koch, "Herpes simplex virus type 1 targets the MHC class II processing pathway for immune evasion," *The Journal of Immunology*, vol. 171, no. 6, pp. 3075–3083, 2003.

[54] J. M. Silverman and N. E. Reiner, "Exosomes and other microvesicles in infection biology: organelles with unanticipated phenotypes," *Cellular Microbiology*, vol. 13, no. 1, pp. 1–9, 2011.

[55] B. György, T. G. Szabó, M. Pásztói et al., "Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles," *Cellular and Molecular Life Sciences*, vol. 68, no. 16, pp. 2667–2688, 2011.

[56] D. G. Meckes Jr. and N. Raab-Traub, "Microvesicles and viral infection," *Journal of Virology*, vol. 85, no. 24, pp. 12844–12854, 2011.

[57] G. Raposo, H. W. Nijman, W. Stoorvogel et al., "B lymphocytes secrete antigen-presenting vesicles," *Journal of Experimental Medicine*, vol. 183, no. 3, pp. 1161–1172, 1996.

[58] A. De Gassart, B. Trentin, M. Martin et al., "Exosomal sorting of the cytoplasmic domain of bovine leukemia virus TM Env protein," *Cell Biology International*, vol. 33, no. 1, pp. 36–48, 2009.

[59] N. Plazolles, J. M. Humbert, L. Vachot, B. Verrier, C. Hocke, and F. Halary, "Pivotal Advance: the promotion of soluble DC-SIGN release by inflammatory signals and its enhancement of cytomegalovirus-mediated cis-infection of myeloid dendritic cells," *Journal of Leukocyte Biology*, vol. 89, no. 3, pp. 329–342, 2011.

[60] F. Masciopinto, C. Giovani, S. Campagnoli et al., "Association of hepatitis C virus envelope proteins with exosomes," *European Journal of Immunology*, vol. 34, no. 10, pp. 2834–2842, 2004.

[61] A. K. Khatua, H. E. Taylor, J. E. K. Hildreth, and W. Popik, "Exosomes packaging APOBEC3G confer human immunodeficiency virus resistance to recipient cells," *Journal of Virology*, vol. 83, no. 2, pp. 512–521, 2009.

[62] Y. V. Liu, M. J. Massare, D. L. Barnard et al., "Chimeric severe acute respiratory syndrome coronavirus (SARS-CoV) S glycoprotein and influenza matrix 1 efficiently form virus-like particles (VLPs) that protect mice against challenge with SARS-CoV," *Vaccine*, vol. 18, pp. 285–294, 2011.

[63] J. Hardcastle, K. Kurozumi, N. Dmitrieva et al., "Enhanced antitumor efficacy of vasculostatin (Vstat120) expressing oncolytic HSV-1," *Molecular Therapy*, vol. 18, no. 2, pp. 285–294, 2010.

[64] J. Y. Yoo, A. Haseley, A. Bratasz et al., "Antitumor efficacy of 34.5ENVE: a transcriptionally retargeted and vstat120-expressing oncolytic virus," *Molecular Therapy*, vol. 20, no. 2, pp. 287–297, 2012.

[65] E. A. Chiocca, "Oncolytic viruses," *Nature Reviews Cancer*, vol. 2, no. 12, pp. 938–950, 2002.

[66] C. A. Maguire, L. Balaj, S. Sivaraman et al., "Microvesicle-associated AAV vector as a novel gene delivery system," *Molecular Therapy*, vol. 20, no. 5, pp. 960–971, 2012.

[67] J. L. Umbach, M. F. Kramer, I. Jurak, H. W. Karnowski, D. M. Coen, and B. R. Cullen, "MicroRNAs expressed by herpes simplex virus 1 during latent infection regulate viral mRNAs," *Nature*, vol. 454, no. 7205, pp. 780–783, 2008.

[68] D. M. Pegtel, M. D. B. van de Garde, and J. M. Middeldorp, "Viral miRNAs exploiting the endosomal-exosomal pathway for intercellular cross-talk and immune evasion," *Biochimica et Biophysica Acta*, vol. 1809, no. 11-12, pp. 715–721, 2011.

[69] F. J. Verweij, M. A. J. Van Eijndhoven, E. S. Hopmans et al., "LMP1 association with CD63 in endosomes and secretion via exosomes limits constitutive NF-κB activation," *The EMBO Journal*, vol. 30, no. 11, pp. 2115–2129, 2011.

[70] T. C. Mettenleiter, "Budding events in herpesvirus morphogenesis," *Virus Research*, vol. 106, no. 2, pp. 167–180, 2004.

[71] Y. Mori, M. Koike, E. Moriishi et al., "Human herpesvirus-6 induces MVB formation, and virus egress occurs by an exosomal release pathway," *Traffic*, vol. 9, no. 10, pp. 1728–1742, 2008.

[72] D. M. Pegtel, J. Middeldorp, and D. A. Thorley-Lawson, "Epstein-barr virus infection in ex vivo tonsil epithelial cell cultures of asymptomatic carriers," *Journal of Virology*, vol. 78, no. 22, pp. 12613–12624, 2004.

[73] V. Hadinoto, M. Shapiro, C. C. Sun, and D. A. Thorley-Lawson, "The dynamics of EBV shedding implicate a central role for epithelial cells in amplifying viral output," *PLoS Pathogens*, vol. 5, no. 7, Article ID e1000496, 2009.

[74] J. E. Roughan, C. Torgbor, and D. A. Thorley-Lawson, "Germinal center B cells latently infected with Epstein-Barr virus proliferate extensively but do not increase in number," *Journal of Virology*, vol. 84, no. 2, pp. 1158–1168, 2010.

[75] J. E. Roughan and D. A. Thorley-Lawson, "The intersection of Epstein-Barr virus with the germinal center," *Journal of Virology*, vol. 83, no. 8, pp. 3968–3976, 2009.

[76] J. P. Pereira, L. M. Kelly, Y. Xu, and J. G. Cyster, "EBI2 mediates B cell segregation between the outer and centre follicle," *Nature*, vol. 460, no. 7259, pp. 1122–1126, 2009.

[77] D. A. Thorley-Lawson and A. Gross, "Persistence of the Epstein-Barr virus and the origins of associated lymphomas," *The New England Journal of Medicine*, vol. 350, no. 13, pp. 1328–1337, 2004.

[78] A. D. Hislop, G. S. Taylor, D. Sauce, and A. B. Rickinson, "Cellular responses to viral infection in humans: lessons from Epstein-Barr virus," *Annual Review of Immunology*, vol. 25, pp. 587–617, 2007.

[79] D. F. Dukers, P. Meij, M. B. H. J. Vervoort et al., "Direct immunosuppressive effects of EBV-encoded latent membrane protein 1," *The Journal of Immunology*, vol. 165, no. 2, pp. 663–670, 2000.

[80] J. Li, X. H. Zeng, H. Y. Mo et al., "Functional inactivation of EBV-specific T-lymphocytes in nasopharyngeal carcinoma: implications for tumor immunotherapy," *PLoS ONE*, vol. 2, no. 11, Article ID e1122, 2007.

[81] H. Vallhov, C. Gutzeit, S. M. Johansson et al., "Exosomes containing glycoprotein 350 released by EBV-transformed B cells selectively target B cells through CD21 and block EBV infection in vitro," *The Journal of Immunology*, vol. 186, no. 1, pp. 73–82, 2011.

[82] R. Ruiss, S. Jochum, R. Mocikat, W. Hammerschmidt, and R. Zeidler, "EBV-gp350 confers B-cell tropism to tailored exosomes is a neo-antigen in normal and malignant B cells-a new option for the treatment of B-CLL," *PLoS ONE*, vol. 6, no. 10, Article ID e25294, 2011.

[83] S. Pfeffer, M. Zavolan, F. A. Grässer et al., "Identification of virus-encoded microRNAs," *Science*, vol. 304, no. 5671, pp. 734–736, 2004.

[84] X. Cai, A. Schäfer, S. Lu et al., "Epstein-Barr virus microRNAs are evolutionarily conserved and differentially expressed," *PLoS Pathogens*, vol. 2, no. 3, article e23, 2006.

[85] R. L. Skalsky, D. L. Corcoran, E. Gottwein et al., "The viral and cellular microRNA targetome in lymphoblastoid cell lines," *PLoS Pathogens*, vol. 8, no. 1, Article ID e1002484, 2012.

[86] S. H. Kim, E. R. Lechman, N. Bianco et al., "Exosomes derived from IL-10-treated dendritic cells can suppress inflammation and collagen-induced arthritis," *The Journal of Immunology*, vol. 174, no. 10, pp. 6440–6448, 2005.

[87] D. G. Meckes Jr., K. H. Y. Shair, A. R. Marquitz, C. P. Kung, R. H. Edwards, and N. Raab-Traub, "Human tumor virus utilizes exosomes for intercellular communication," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 47, pp. 20370–20375, 2010.

[88] M. I. Yuseff, A. Reversat, D. Lankar et al., "Polarized secretion of lysosomes at the B cell synapse couples antigen extraction to processing and presentation," *Immunity*, vol. 35, pp. 361–374, 2011.

[89] N. Izquierdo-Useros, M. C. Puertas, F. E. Borràs, J. Blanco, and J. Martinez-Picado, "Exosomes and retroviruses: the chicken or the egg?" *Cellular Microbiology*, vol. 13, no. 1, pp. 10–17, 2011.

[90] N. Izquierdo-Useros, M. Naranjo-Gómez, I. Erkizia et al., "HIV and mature dendritic cells: trojan exosomes riding the Trojan horse?" *PLoS Pathogens*, vol. 6, no. 3, Article ID e1000740, 2010.

[91] N. Onlamoon, K. Pattanapanyasat, and A. A. Ansari, "Human and nonhuman primate lentiviral infection and autoimmunity," *Annals of the New York Academy of Sciences*, vol. 1050, pp. 397–409, 2005.

[92] S. J. Gould, A. M. Booth, and J. E. K. Hildreth, "The Trojan exosome hypothesis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 19, pp. 10592–10597, 2003.

[93] X. Gan and S. J. Gould, "Identification of an inhibitory budding signal that blocks the release of HIV particles and exosome/microvesicle proteins," *Molecular Biology of the Cell*, vol. 22, no. 6, pp. 817–830, 2011.

[94] M. N. Shelton, M.-B. Huang, S. A. Ali, M. D. Powell, and V. C. Bond, "Secretion modification region-derived peptide disrupts HIV-1 Nef's interaction with mortalin and blocks virus and Nef exosome release," *Journal of Virology*, vol. 86, no. 1, pp. 406–419, 2012.

[95] S. Popov, E. Popova, M. Inoue, and H. G. Göttlinger, "Human immunodeficiency virus type 1 Gag engages the Bro1 domain of ALIX/AIP1 through the nucleocapsid," *Journal of Virology*, vol. 82, no. 3, pp. 1389–1398, 2008.

[96] Y. Fang, N. Wu, X. Gan, W. Yan, J. C. Morrell, and S. J. Gould, "Higher-order oligomerization targets plasma membrane proteins and HIV gag to exosomes," *PLoS Biology*, vol. 5, no. 6, article e158, 2007.

[97] A. M. Booth, Y. Fang, J. K. Fallon et al., "Exosomes and HIV Gag bud from endosome-like domains of the T cell plasma membrane," *Journal of Cell Biology*, vol. 172, no. 6, pp. 923–935, 2006.

[98] N. Jouvenet, S. M. Simon, and P. D. Bieniasz, "Visualizing HIV-1 assembly," *Journal of Molecular Biology*, vol. 410, no. 4, pp. 501–511, 2011.

[99] N. Jouvenet, M. Zhadina, P. D. Bieniasz, and S. M. Simon, "Dynamics of ESCRT protein recruitment during retroviral assembly," *Nature Cell Biology*, vol. 13, no. 4, pp. 394–402, 2011.

[100] V. Baumgärtel, S. Ivanchenko, A. Dupont et al., "Live-cell visualization of dynamics of HIV budding site interactions with an ESCRT component," *Nature Cell Biology*, vol. 13, no. 4, pp. 469–476, 2011.

[101] P. D. Bieniasz, "The cell biology of HIV-1 virion genesis," *Cell Host and Microbe*, vol. 5, no. 6, pp. 550–558, 2009.

[102] A. Pelchen-Matthews, G. Raposo, and M. Marsh, "Endosomes, exosomes and Trojan viruses," *Trends in Microbiology*, vol. 12, no. 7, pp. 310–316, 2004.

[103] I. W. Park and J. J. He, "HIV-1 is budded from $CD4^+$ T lymphocytes independently of exosomes," *Virology Journal*, vol. 7, article 234, 2010.

[104] D. G. Nguyen, A. Booth, S. J. Gould, and J. E. K. Hildreth, "Evidence that HIV budding in primary macrophages occurs through the exosome release pathway," *The Journal of Biological Chemistry*, vol. 278, no. 52, pp. 52347–52354, 2003.

[105] N. Jouvenet, S. J. Neil, C. Bess et al., "Plasma membrane is the site of productive HIV-1 particle assembly," *PLoS Biology*, vol. 4, no. 12, article e435, 2006.

[106] S. Welsch, O. T. Keppler, A. Habermann, I. Allespach, J. Krijnse-Locker, and H. G. Kräusslich, "HIV-1 buds predominantly at the plasma membrane of primary human macrophages," *PLoS Pathogens*, vol. 3, no. 3, article e36, 2007.

[107] P. Benaroch, E. Billard, R. Gaudin, M. Schindler, and M. Jouve, "HIV-1 assembly in macrophages," *Retrovirology*, vol. 7, article 29, 2010.

[108] M. Deneka, A. Pelchen-Matthews, R. Byland, E. Ruiz-Mateos, and M. Marsh, "In macrophages, HIV-1 assembles into an intracellular plasma membrane domain containing the tetraspanins CD81, CD9, and CD53," *Journal of Cell Biology*, vol. 177, no. 2, pp. 329–341, 2007.

[109] G. Raposo, B. Fevrier, W. Stoorvogel, and M. S. Marks, "Lysosome-related organelles: a view from immunity and pigmentation," *Cell Structure and Function*, vol. 27, no. 6, pp. 443–456, 2002.

[110] D. S. Kwon, G. Gregorio, N. Bitton, W. A. Hendrickson, and D. R. Littman, "DC-SIGN-mediated internalization of HIV is required for trans-enhancement of T cell infection," *Immunity*, vol. 16, no. 1, pp. 135–144, 2002.

[111] N. Izquierdo-Useros, M. Naranjo-Gómez, J. Archer et al., "Capture and transfer of HIV-1 particles by mature dendritic cells converges with the exosome-dissemination pathway," *Blood*, vol. 113, no. 12, pp. 2732–2741, 2009.

[112] M. Cavrois, J. Neidleman, J. F. Kreisberg, and W. C. Greene, "In vitro derived dendritic cells trans-infect CD4 T cells primarily with surface-bound HIV-1 virions," *PLoS Pathogens*, vol. 3, no. 1, article e4, 2007.

[113] M. Cavrois, J. Neidleman, and W. C. Greene, "The Achilles Heel of the Trojan horse model of HIV-1 trans-infection," *PLoS Pathogens*, vol. 4, no. 6, Article ID e1000051, 2008.

[114] H. J. Yu, M. A. Reuter, and D. McDonald, "HIV traffics through a specialized, surface-accessible intracellular compartment during trans-infection of T cells by mature dendritic cells," *PLoS Pathogens*, vol. 4, no. 8, Article ID e1000134, 2008.

[115] B. Brügger, B. Glass, P. Haberkant, I. Leibrecht, F. T. Wieland, and H. G. Kräusslich, "The HIV lipidome: a raft with an unusual composition," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 8, pp. 2641–2646, 2006.

[116] L. Krishnamoorthy, J. W. Bess, A. B. Preston, K. Nagashima, and L. K. Mahal, "HIV-1 and microvesicles from T cells share a common glycome, arguing for a common origin," *Nature Chemical Biology*, vol. 5, no. 4, pp. 244–250, 2009.

[117] P. A. Holme, F. Müller, N. O. Solum, F. Brosstad, S. S. FrØland, and P. Aukrust, "Enhanced activation of platelets with abnormal release of RANTES in human immunodeficiency virus type 1 infection," *The FASEB Journal*, vol. 12, no. 1, pp. 79–89, 1998.

[118] P. Stumptner-Cuvelette, M. Jouve, J. Helft et al., "Human immunodeficiency virus-1 Nef expression induces intracellular accumulation of multivesicular bodies and major histocompatibility complex class II complexes: potential role of phosphatidylinositol 3-kinase," *Molecular Biology of the Cell*, vol. 14, no. 12, pp. 4857–4870, 2003.

[119] L. J. Costa, N. Chen, A. Lopes et al., "Interactions between Nef and AIP1 proliferate multivesicular bodies and facilitate egress of HIV-1," *Retrovirology*, vol. 3, article 33, 2006.

[120] R. Madrid, K. Janvier, D. Hitchin et al., "Nef-induced alteration of the early/recycling endosomal compartment correlates with enhancement of HIV-1 infectivity," *The Journal of Biological Chemistry*, vol. 280, no. 6, pp. 5032–5044, 2005.

[121] A. Sanfridson, S. Hester, and C. Doyle, "Nef proteins encoded by human and simian immunodeficiency viruses induce the accumulation of endosomes and lysosomes in human T cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 3, pp. 873–878, 1997.

[122] J. L. Goodier and H. H. Kazazian, "Retrotransposons revisited: the restraint and rehabilitation of parasites," *Cell*, vol. 135, no. 1, pp. 23–35, 2008.

[123] K. H. Burns and J. D. Boeke, "Great exaptations," *Journal of Biology*, vol. 7, no. 2, article 5, 2008.

[124] C. Esnault, J. Maestre, and T. Heidmann, "Human LINE retrotransposons generate processed pseudogenes," *Nature Genetics*, vol. 24, no. 4, pp. 363–367, 2000.

[125] K. Ruprecht, H. Ferreira, A. Flockerzi et al., "Human endogenous retrovirus family HERV-K(HML-2) RNA transcripts are selectively packaged into retroviral particles produced by the human germ cell tumor line tera-1 and originate mainly from a provirus on chromosome 22q11.21," *Journal of Virology*, vol. 82, no. 20, pp. 10008–10016, 2008.

[126] X. Song, A. Sui, and A. Garen, "Binding of mouse VL30 retrotransposon RNA to PSF protein induces genes repressed by PSF: effects on steroidogenesis and oncogenesis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 2, pp. 621–626, 2004.

[127] S. Boissinot, P. Chevret, and A. V. Furano, "L1 (LINE-1) retrotransposon evolution and amplification in recent human history," *Molecular Biology and Evolution*, vol. 17, no. 6, pp. 915–928, 2000.

[128] M. K. Konkel, J. Wang, P. Liang, and M. A. Batzer, "Identification and characterization of novel polymorphic LINE-1 insertions through comparison of two human genome sequence assemblies," *Gene*, vol. 390, no. 1-2, pp. 28–38, 2007.

[129] I. Martínez-Garay, M. J. Ballesta, S. Oltra et al., "Intronic L1 insertion and F268S, novel mutations in RPS6KA3 (RSK2) causing Coffin-Lowry syndrome," *Clinical Genetics*, vol. 64, no. 6, pp. 491–496, 2003.

[130] J. A. J. M. Van Den Hurk, D. J. R. Van De Pol, B. Wissinger et al., "Novel types of mutation in the choroideremia (CHM) gene: a full-length L1 insertion and an intronic mutation

activating a cryptic exon," *Human Genetics*, vol. 113, no. 3, pp. 268–275, 2003.

[131] P. A. Callinan and M. A. Batzer, "Retrotransposable elements and human disease," *Genome Dynamics*, vol. 1, pp. 104–115, 2006.

[132] R. C. Iskow, M. T. McCabe, R. E. Mills et al., "Natural mutagenesis of human genomes by endogenous retrotransposons," *Cell*, vol. 141, no. 7, pp. 1253–1261, 2010.

[133] D. J. Griffiths, "Endogenous retroviruses in the human genome sequence," *Genome Biology*, vol. 2, no. 6, article 1017, 2001.

[134] K. Boller, K. Schönfeld, S. Lischer et al., "Human endogenous retrovirus HERV-K113 is capable of producing intact viral particles," *Journal of General Virology*, vol. 89, no. 2, pp. 567–572, 2008.

[135] B. K. Prusty, H. zur Hausen, R. Schmidt, R. Kimmel, and E. M. de Villiers, "Transcription of HERV-E and HERV-E-related sequences in malignant and non-malignant human haematopoietic cells," *Virology*, vol. 382, no. 1, pp. 37–45, 2008.

[136] L. Lavie, M. Kitova, E. Maldener, E. Meese, and J. Mayer, "CpG methylation directly regulates transcriptional activity of the human endogenous retrovirus family HERV-K(HML-2)," *Journal of Virology*, vol. 79, no. 2, pp. 876–883, 2005.

[137] W. Seifarth, H. Skladny, F. Krieg-Schneider, A. Reichert, R. Hehlmann, and C. Leib- Mosch, "Retrovirus-like particles released from the human breast cancer cell line T47-D display type B- and C-related endogenous retroviral sequences," *Journal of Virology*, vol. 69, no. 10, pp. 6408–6416, 1995.

[138] D. L. Bronson, E. E. Fraley, J. Fogh, and S. S. Kalter, "Induction of retrovirus particles in human testicular tumor (Tera-1) cell cultures: an electron microscopic study," *Journal of the National Cancer Institute*, vol. 63, no. 2, pp. 337–339, 1979.

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Review Article

Human Herpesviridae Methods of Natural Killer Cell Evasion

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Human herpesviruses cause diseases of considerable morbidity and mortality, ranging from encephalitis to hematologic malignancies. As evidence emerges about the role of innate immunity and natural killer (NK) cells in the control of herpesvirus infection, evidence of viral methods of innate immune evasion grows as well. These methods include interference with the ligands on infected cell surfaces that bind NK cell activating or inhibitory receptors. This paper summarizes the most extensively studied NK cell receptor/ligand pairs and then describes the methods of NK cell evasion used by all eight herpesviruses through these receptors and ligands. Although great strides have been made in elucidating their mechanisms, there is still a disparity between viruses in the amount of knowledge regarding innate immune evasion. Further research of herpesvirus innate immune evasion can provide insight for circumventing viral mechanisms in future therapies.

1. Introduction (Herpesviridae and Disease)

The human herpes family of viruses includes human cytomegalovirus (HCMV), Kaposi's sarcoma herpesvirus (KSHV), herpes simplex virus types 1 and 2 (HSV-1, 2), varicella zoster virus (VZV), Epstein-Barr virus (EBV), and human herpesvirus 6 and 7 (HHV6, 7). These viruses share similar characteristics: all contain linear double-stranded DNA, are enveloped, and undergo latent and lytic lifecycles. However, there are important differences between these viruses in terms of infection niche and immune evasion strategies for persistent infection.

Herpesviridae evasion of adaptive immune responses has been previously described [1–4]. This paper will focus on herpesvirus innate immune evasion, specifically viral evasion of the natural killer (NK) cells response. Reviews on broad interactions between viruses and NK cells can be found in references [5–8]. The role of NK cells in controlling herpes viral infections become apparent in consideration that multiple herpes infections have been documented in patients lacking NK cells [9] and evidence of NK activation during viral infection [10–13].

2. NK Cells and Activation

NK cells are important innate immune cells involved in the regulation of viral infection [14, 15]. They are a lymphocyte subset of the innate immune system that kills without prior exposure and sensitization to antigens via release of granzymes, perforin, TRAIL, and FAS ligand [16]. NK cells are regulated through surface receptor interactions with ligands expressed on stressed cells, such as virally infected or malignantly transformed cells. NK cells possess both activating and inhibitory cell surface receptors; it is the balance of ligand interactions with these receptors that determine NK cell activation. The structures, functions, and signaling mechanisms of these receptors and their ligands are comprehensively reviewed in references [16–21]. In addition to receptor-mediated regulation, cytokines induced during viral infection (IL-15, IL-12, IL-8, IFN-α, and IFN-β) can

indirectly activate NK cells as well [6]. A summary of the receptors present on NK cells and associated ligands most relevant to immune evasion by human herpesviruses is provided below.

2.1. Activating Receptors and Ligands

2.1.1. Natural Killer Group 2 Member D (NKG2D) Receptor. NKG2D is a receptor found prominently on NK cells that provides activation signals through the coreceptor DAP-10 upon ligand binding. The ligands that bind NKG2D include (1) the MHC-I-like molecules MHC-class-I-polypeptide-related sequence A (MICA) and B (MICB), (2) UL16 binding proteins (ULBP1–4 and 6), and (3) retinoic acid early transcript 1G (RAET1G). This interaction with multiple activating ligands is unique to NKG2D and does not occur with the other NK cell activating receptors [22, 23]. Investigators have proposed that this development of multiple activating ligands is a coevolutionary responses to viral or tumor pressure [22]. The structures of MICA and MICB are similar to MHC-I with alpha domains; however, they do not engage β2-microglobulin [24–26]. Surface expression of these ligands is normally absent or low on healthy cells and increases upon events of cellular stress such as viral infection, DNA damage, oxidative stress, and oncogenic stress [22, 27–30]. MICA is noted to have a large polymorphic distribution, with over 73 alleles identified [31]. A subset group of MICA alleles contains a frameshift mutation resulting in a premature stop codon and subsequent truncation of the cytoplasmic C-terminus. Interestingly, the MICA allele $*008$ encodes a truncated protein and is the most frequently distributed MICA allele in various populations across the world [32–39]. ULBP1–4 and RAET1 have alpha1 and alpha2 domains similar to MICA/B; however, unlike MICA/B, they do not contain alpha3 domains and their mRNA is expressed at low levels even in normal cells without corresponding surface expression [23, 40].

2.1.2. Natural Cytotoxicity Receptors (NCRs). The NCRs contain immunoglobulin (Ig)-like domains and include NKp30, NKp44, NKp46, and NKp80 [41, 42]. A role for NCRs has been implicated in the prognosis of leukemia [43, 44] and the recognition/killing of various solid tumors [45, 46]. Only NKp30 has a confirmed ligand, the tumor ligand B7-H6 [47, 48]. Additional ligands for the NCRs are unknown, although possible ligands have been identified and include nuclear factor BAT3 [49] and a number of viral hemagglutinin proteins and heparan sulfate structures [50, 51].

2.1.3. DNAX Accessory Molecule-1 (DNAM-1). DNAM-1 is a member of the Ig super family that recognizes CD112 (nectin-2) and the poliovirus receptor [17]. Similar to other activating receptors, there is expression of DNAM-1 ligands on various tumors resulting in DNAM-1-mediated killing alone or in concert with other receptors [52–56]. Aberrations in DNAM-1 expression or DNAM-1 expressing NK cells have also been linked to a variety of autoimmune diseases [57–59].

2.2. Inhibitory Receptors and Ligands. The primary inhibitory receptors include the killer Ig-like inhibitory receptors (KIRs) and CD94-NKG2A lectin-like inhibitory receptor. The KIRs and CD94-NKG2A bind to MHC-I molecules and diminish NK cell activation. There has been no evidence to date for their binding to MHC-II molecules. The receptor-ligand interactions for both KIRs and CD94-NKG2A are MHC-I isotype specific [60–62]. In accordance with the "Missing Self" hypothesis first proposed by Karre et al., the lack of MHC-I on target cells removes the inhibitory signals from NK cells, thus leading to unopposed activation [63, 64].

2.2.1. Killer Ig-Like Inhibitory Receptors (KIRs). The KIRs are members of the Ig superfamily that recognize MHC-I molecules of the HLA-C isotype on surrounding cells [17, 19]. The absence of HLA-C on tumor and virus infected cells can result in loss of NK cell inhibition [21, 65, 66].

2.2.2. Leukocyte Ig-Like Receptor (LIR)-1. Like the KIRs, LIR-1 contains Ig domains and binds MHC-I, but with a lower affinity than other inhibitory receptors [17, 19]. LIR-1 expression is more variable on NK cells than other immune cells [17].

2.2.3. CD94-NKG2A Lectin-Like Inhibitory Receptor. This receptor is a C-lectin-like heterodimer that recognizes MHC-I molecules of the HLA-E isotype. Ligation of HLA-E by CD94-NKG2A leads to inhibition of NK cells, yet HLA-E ligation can activate NK cells if CD94 is complexed to NKG2C or -E [17, 19]. Similar to KIRs, the CD94-NKG2A complex results in loss of NK cell inhibition in the absence of HLA-E. However, the uninhibited activity is not as strong as that mediated by KIRs [65].

3. Herpesviridae Methods of NK Cell Evasion

Human herpesviruses have evolved multiple mechanisms to dampen NK cell cytotoxicity, interacting with many of the factors influencing the balance of NK cell activation and inhibition. A summary of these mechanisms is provided in Table 1. A number of methods employed by human herpesviruses hinder the expression of NK cell ligands on infected cells. This method of immune evasion has been studied in different members of the herpesvirus family, defining marked similarities and stark differences between family members. Multiple mechanisms offset the indirect NK cell activation prompted by lack of MHC-I surface expression. As many human herpesviruses diminish MHC-I presentation of viral antigens to avoid detection by cytotoxic T lymphocytes, these mechanisms may offset the loss of NK cell inhibition from "Missing Self" [64, 67].

3.1. CMV. The HCMV product UL18 is an MHC-I homologue that binds the inhibitory NK cell receptor LIR, possibly as a means of increasing the inhibitory signal [80, 81]. However, inhibition via this ortholog is controversial [101–103]. CMV also encodes UL40, which stabilizes and promotes surface expression of the HLA-E isotype. This

Table 1: Summary of known interactions between NK cell receptors, ligands, and herpesvirus immunoevasins.

Major receptors	Ligand	Virus	Immunoevasin	Mechanism	References
Activating					
NKG2D	MICA	HCMV	UL142	Internal retention	[68, 69]
		KSHV	K5	Ubiquitination/sequestration	[70, 71]
		HSV	?	?	[72, 73]
		HHV-7	U21	?	[74]
	MICB	HCMV	UL16	Internal retention	[23, 75–77]
		HCMV	miR-UL112	Translational downregulation	[78]
		KSHV	K5	Ubiquitination/sequestration	[70, 71]
		KSHV	miRK12-7	Translational downregulation	[79]
		HSV	?	?	[72, 73]
		EBV	miR-BART2-5p	Translational downregulation	[79]
		HHV-7	U21	?	[74]
	ULBP1–4	HCMV	UL16	Internal retention	[23, 75–77]
		HSV	?	?	[72]
		HHV-7	U21	Lysosomal degradation	[74]
NCRs	AICL	KSHV	K5		[71]
DNAM-1	PVR	?	?		
	CD112	?	?		
Inhibitory					
LIR-1, KIRs, CD94/NKG2A	MHC-I	HCMV	UL18	MHC-I homologue	[80, 81]
		HCMV	UL40	Signal prolongation	[82–84]
		HCMV	US2, US3, US6, US11	Retention/degradation	[64, 67]
		KSHV	K5, K3	Endocytosis	[85–87]
		HSV	ICP47	TAP interference	[88–90]
		EBV	vIL-10	IL-10 homolog	[91]
		EBV	BNLF2a	TAP interference	[92, 93]
		EBV	BILF1	Endocytosis/degradation	[94]
		HHV-6	U21 analogues	Lysosomal degradation	[95]
		HHV-7	U21	Lysosomal degradation	[96–98]
		VZV	ORF66	Internal retention	[99, 100]

diminishes NK cell activation by increased ligation of the CD94-NKG2A receptor [82–84]. US11 targets HLA-A; US2 and US3 target HLA-A and HLA-G while sparing HLA-E; US6 targets HLA-A,G, and E for degradation to diminish cytotoxic T-cell detection [64, 67, 104].

In addition to inducing an inhibitory response, HCMV also suppresses activating ligands that bind NKG2D. HCMV UL16 binds MICB, ULBP1, and ULBP2 to sequester these proteins in the ER of infected cells but is unable to bind to RAET1G [23, 75–77, 105]. The crystal structure of UL16-MICB complex has been characterized in reference [106]. The HCMV protein UL142 blocks surface expression of some MICA alleles by interacting with the cytosolic carboxyl-terminal region of the transmembrane protein and retaining it in the golgi network, limiting surface expression of the activating ligand. HCMV UL142 cannot downmodulate truncated forms of MICA lacking the intracellular carboxy terminus. It is interesting that, of the >70 MICA allelic forms, the MICA∗008 truncated form is present in a majority of the population and may provide a selective advantage [68, 70]. There is also evidence that HCMV encodes the microRNA mIR-UL112 that decreases MICB production to escape NKG2D detection [78, 107].

In summary, HCMV has multiple means of NK cell ligand manipulation. UL18 is a mock MHC-I molecule that takes advantage of NK cell inhibitory receptors, while UL40 prolongs the inhibitory signals of actual host MHC-I molecules. UL16 retains NKG2D ligands (except MICA and ULBP3) to prevent activation, while UL142 downmodulates MICA in an allele-dependent manner. All of the methods of immune evasion used by CMV are more comprehensively reviewed in references [2, 108].

3.2. KSHV. KSHV encodes proteins that target MHC-I to prevent viral antigen presentation to T-lymphocytes as does HCMV; however, the molecular mechanisms differ. KSHV K3 and K5 are E3 ubiquitin ligases that transfer ubiquitin to the cytoplasmic tails of proteins [70, 85]. The K5 protein

targets HLA-A and HLA-B for endocytosis from cell surfaces, while the K3 protein targets HLA-A, B, C, and E [85–87]. No interactions with HLA-G are known.

Like UL142 of CMV, the KSHV protein K5 also blocks surface expression of MICA and MICB but is unable to downregulate the MICA∗008 allele due to the absence of a cytoplasmic tail and lysine ubiquitin sites [71]. Ubiquitinated MICA proteins are endocytosed from the infected cell surface and retained in cytoplasmic vesicles without increased degradation [71]. K5-mediated downmodulation protects infected cells from NK cell cytotoxicity [71]. K5 also downmodulates the activating ligands B7-2, AICL, and ICAM-1 by a similar mechanism [71, 109]. In contrast to acute lytic infection, chronic infection with KSHV results in higher levels of MHC-I, MICA/B, PVR, and CD112 expression [110]. Akin to HCMV, KSHV encodes the microRNA miRK-12-7 inhibiting MICB expression [79]. Additionally, KSHV has been reported to infect NK cells, leading to downmodulation of the activating NCRs and NKG2D receptors on NK cell surfaces [110].

To summarize, KSHV is similar to CMV in that both viruses encode proteins downmodulating MHC-I and MICA/B. The mechanism by which KSHV proteins function diverges from HCMV proteins in that KSHV K3 and K5 ubiquinate and promote endocytosis of targeted proteins, whereas HCMV UL142 and UL16 prevent protein maturation and surface expression. Similar to HCMV UL142, KSHV K5 downmodulation of MICA is allele dependent. The mechanistic basis is the absence of ubiquitin sites in the truncated cytoplasmic tail. To date, there is no evidence of KSHV mechanisms affecting the expression of the ULBP or RAET ligands. KSHV specific immune evasion is reviewed in more detail in [70, 111].

3.3. HSV-1 and 2. The exact mechanisms by which HSV-1 modulates NK cell inhibitory and activating ligands are less studied than those for HCMV and KSHV. The HSV-1 and 2 US12 gene product (infected cell protein 47, ICP47) downmodulates MHC-I surface expression by suppressing MHC-I transport from the ER [64, 72]. ICP47 binds to the transporter associated with antigen presentation (TAP) and in doing so inhibits MHC-I antigen loading and expression of antigenic peptides generated by proteasomal degradation that then translocate from the cytosol to the ER lumen [88–90, 112]. Cells engineered to express ICP47 failed to express antigenic peptides [90]. Interestingly, HSV-1 induces expression of certain HLA-G isoforms while decreasing the surface expression of others [113].

The consequences of MHC-I downmodulation on NK cell recognition of HSV-1 and HSV-2 infected cells are controversial. Studies using antibody blocking of KIRs and MHC-I in conjunction with exogenous ICP47 expression suggest that the protective properties of MHC-I via KIR inhibitory signaling are rendered ineffective upon infection with HSV-1 [64]. However, some studies utilizing ICP47 deleted recombinant HSV-1 and anti-KIR antibodies suggest that NK cell inhibitory effects of MHC-I molecules are not significant enough alone to diminish cytotoxicity and that the viral product ICP47 is not necessary in inducing susceptibility to NK cell killing [73]. There are yet other findings that suggest a qualitative change in MHC-I molecules, such as the binding site shape presented to NK cells during HSV infection rather than the quantity of molecules presented, may contribute to NK recognition and killing [114].

There are few studies examining the influence of HSV-1 and HSV-2 infection upon the NK cell activating ligands. Experiments utilizing HSV-1 recombinants deleted of all IE genes except for ICP0 were able to induce NK cell induced lysis of human fibroblasts. Fibroblasts expressing ICP0 yielded similar results [73]. Infection with these recombinants also demonstrated an upregulation of unknown ligands binding to NCRs with cytotoxicity dependent upon their presence, yet this only occurred at low multiplicities of infection (MOIs). ICP0-independent mechanisms were reported at higher MOIs [73]. In contrast, studies of HSV effects on NKG2D ligands demonstrated decreased surface expression of MICA in HeLa and U373 cells infected with HSV-1 or HSV-2 with no difference in total protein levels [72]. Interestingly, this HSV-mediated downmodulation of MICA occurs with both the full-length and the truncated protein encoded by the MICA∗008 allele. This diverges significantly from the inability of HCMV and KSHV to downmodulate truncated MICA variants. Down-modulation of MICA was reported with ICP0 deleted recombinant HSV but not with PAA blocking of late gene expression, suggestive that this phenomenon is dependent on late-gene expression [72].

These studies together suggest HSV-1 and HSV-2 employ both early and late gene modulation of NK activating ligands, each with potentially different consequences for virus-infected cells. ICP0 might be sufficient to trigger NK cell cytotoxicity at low MOIs through upregulation of NCR ligands, which would be deleterious for virus survival. Yet at higher MOIs mechanisms other than ICP0 contribute to infected cell susceptibility. MICA downmodulation was shown to be independent of ICP0 expression and may be caused by late gene products. As posited by Schepis et al., HSV-1 may cause infected cells to be particularly susceptible to NK cell-mediated killing early in infection due to ICP0 upregulation of NCR ligands while attempting immunoevasion later in infection by NKG2D ligand and MHC-I downregulation [72]. Although HSV-1 and HSV-2 microRNAs have been documented, none have been found to interfere with NK cell pathways.

3.4. EBV. As with the previously described human herpesviruses, EBV has methods of interfering with MHC-I to prevent presentation of viral antigens to cytotoxic T cells. During active B-cell infection, EBV expresses a viral homolog of interleukin-10 (vIL-10) [91] and BNLF2a, a lytic-phase viral protein [92, 93]; both have been reported to downregulate the expression of TAP and in turn decrease surface MHC-I. BILF1 is a protein also expressed during lytic EBV infection that mediates both increased endocytosis/degradation and decreased exocytosis/presentation of MHC-I [94]. Downmodulation of all isotypes of MHC-I during EBV lytic infection, as well as subsequent decrease in inhibitory binding to KIRs and CD94-NKG2A, results

in increased sensitivity to NK cell-mediated killing [115]. Instead of downregulation of activating ligands to offset this decrease in inhibition, the same studies found an increase in ULBP1 and CD112 expression that contributed to NK cell activation [115]. The only reported mechanism to possibly offset this indirect NK cell activation is a microRNA (miR-BART2-5p) inhibiting MICB expression [79]. EBV may possibly interfere with NKG2D activation through down-modulation of the NKG2D receptor itself via indoleamine-2, 3-dioxygenase metabolites, although the functional consequences have yet to be reported [116]. Similar to KSHV, EBV can infect NK cells, causing aberrantly high expression of the inhibitory CD94-NKG2A receptor but diminished expression of the KIRs [117].

3.5. HHV-6 and HHV-7. The involvement of NK cells in the control of HHV-6 and HHV-7 infection has been documented through studies of IL-2 and IL-15 enhancement of cytotoxicity [118–120]. However, the only documented HHV-7 protein involved in NK cell ligand modulation is U21, a transmembrane protein capable of downmodulating both MHC-I and MICA/B. U21 binds and redirects MHC-I trafficking to lysosomal compartments most similarly to HCMV MHC-I interfering proteins [96–98]. The effect on NK cell killing through this method has not been established. U21 also binds to ULBP1 for redirection to lysosomes and decreases surface MICA and MICB by an undefined mechanism that results in decreased NK cell cytotoxicity [74]. The A and B variants of HHV-6 express proteins analogous to U21 that also bind MHC-I for lysosome redirection [95], but the effect on NK cells or identification of mechanisms affecting activating ligands has not been established.

3.6. VZV. Although NK cells have long been implicated in the control of VZV infection [121–123], specific interactions with infected cells through NK cell receptors have not been extensively studied. VZV downregulates MHC-I on infected cell surfaces via the viral protein kinase ORF66, leading to retention of MHC-I molecules in the golgi [99, 100]. However, any functional consequences of ORF66 on NK cell recognition and killing have not been demonstrated. Likewise, no methods of VZV interference with NK cell activating ligands have been reported to date.

4. Conclusion

Human herpesviruses possess multiple mechanisms for evading both innate and adaptive immune responses. A summary of NK cell receptors, their ligands, and viral mechanisms interfering with each is provided in Table 1. A primary point of interest is the diversity of NK cell evasion mechanisms employed by human herpesviruses. Other than mechanisms shared by the highly similar HSV-1 and HSV-2, the human herpesviruses have evolved different mechanisms for subverting the immune response. It is also notable that these immunoevasion mechanisms do not group with herpesvirus subfamilies. For example, HCMV (a betaherpesvirus) and KSHV (a gammaherpesvirus) both encode microRNA's targeting MICB yet downmodulate MICA via different mechanisms. The NK cell evasion mechanisms are unique to each human herpesvirus likely reflecting selection pressures encountered in the various infection niches occupied by the viruses. The lack of well-defined mechanisms of NK cell immunoevasion by given herpesviruses (i.e., HSV and VZV) is puzzling. There is well-documented persistence of HSV in patients with NK cell defects and the importance of NK cell involvement in the control of disease. Continuing research will likely reveal as yet unknown mechanisms of immunoevasion by the alpha herpesviruses.

Human herpesviruses cause substantial morbidity and mortality. HSV-1 is regarded as one of the most common causes of viral encephalitis, an infection carrying significant risk of mortality [124, 125]. EBV, HCMV, and KSHV infections have the potential to not only cause severe manifestations during acute infection, but also the development of hematologic or solid malignancies [126, 127]. A variety of herpesviruses also cause cutaneous and ocular infections with potential for life-long morbidity [128–130]. Thorough knowledge of specific viral immune evasion mechanisms may provide avenues for developing more effective therapies against disease related to human herpesviruses. Understanding NK cell evasion may improve oncolytic herpesvirus therapies for cancer [131–133]. Insight into viral abilities to evade the immune system may also yield better markers for clinical prognosis and monitoring of active and latent infection [117, 134]. Continued research into these mechanisms of NK cell evasion will not only deepen basic understandings of human herpesviruses but may also serve to ultimately alleviate disease burden and guide strategies for clearance of persistent infection in immunocompromised patients.

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References

[1] T. Chan, N. G. Barra, A. J. Lee, and A. A. Ashkar, "Innate and adaptive immunity against herpes simplex virus type 2 in the genital mucosa," *Journal of Reproductive Immunology*, vol. 88, no. 2, pp. 210–218, 2011.

[2] S. E. Jackson, G. M. Mason, and M. R. Wills, "Human cytomegalovirus immunity and immune evasion," *Virus Research*, vol. 157, no. 2, pp. 151–160, 2011.

[3] L. Coscoy, "Immune evasion by Kaposi's sarcoma-associated herpesvirus," *Nature Reviews Immunology*, vol. 7, no. 5, pp. 391–401, 2007.

[4] E. J. Wiertz, R. Devlin, H. L. Collins, and M. E. Ressing, "Herpesvirus interference with major histocompatibility complex class II-restricted T-cell activation," *Journal of Virology*, vol. 81, no. 9, pp. 4389–4396, 2007.

[5] S. Jonjić, M. Babić, B. Polić, and A. Krmpotić, "Immune evasion of natural killer cells by viruses," *Current Opinion in Immunology*, vol. 20, no. 1, pp. 30–38, 2008.

[6] L. L. Lanier, "Evolutionary struggles between NK cells and viruses," *Nature Reviews Immunology*, vol. 8, no. 4, pp. 259–268, 2008.

[7] A. Groth, S. Klöss, E. Pogge Von Strandmann, U. Koehl, and J. Koch, "Mechanisms of tumor and viral immune escape from natural killer cell-mediated surveillance," *Journal of Innate Immunity*, vol. 3, no. 4, pp. 344–354, 2011.

[8] V. J. Lisnić, A. Krmpotić, and S. Jonjić, "Modulation of natural killer cell activity by viruses," *Current Opinion in Microbiology*, vol. 13, no. 4, pp. 530–539, 2010.

[9] C. A. Biron, K. S. Byron, and J. L. Sullivan, "Severe herpesvirus infections in an adolescent without natural killer cells," *The New England Journal of Medicine*, vol. 320, no. 26, pp. 1731–1735, 1989.

[10] P. A. Fitzgerald, M. Mendelsohn, and C. Lopez, "Human natural killer cells limit replication of herpes simplex virus type 1 in vitro," *Journal of Immunology*, vol. 134, no. 4, pp. 2666–2672, 1985.

[11] C. V. Paya, R. A. Schoon, and P. J. Leibson, "Alternative mechanisms of natural killer cell activation during herpes simplex virus infection," *Journal of Immunology*, vol. 144, no. 11, pp. 4370–4375, 1990.

[12] P. Fitzgerald-Bocarsly, D. M. Howell, L. Pettera, S. Tehrani, and C. Lopez, "Immediate-early gene expression is sufficient for induction of natural killer cell-mediated lysis of herpes simplex virus type 1-infected fibroblasts," *Journal of Virology*, vol. 65, no. 6, pp. 3151–3160, 1991.

[13] C. Lopez, D. Kirkpatrick, and P. A. Fitzgerald, "Studies of the cell lineage of the effector cells that spontaneously lyse HSV-1 infected fibroblasts (NK(HSV-1))," *Journal of Immunology*, vol. 129, no. 2, pp. 824–828, 1982.

[14] F. Marras, F. Bozzano, and A. De Maria, "Involvement of activating NK cell receptors and their modulation in pathogen immunity," *Journal of Biomedicine and Biotechnology*, vol. 2011, Article ID 152430, 11 pages, 2011.

[15] S. H. Lee and C. A. Biron, "Here today—not gone tomorrow: roles for activating receptors in sustaining NK cells during viral infections," *European Journal of Immunology*, vol. 40, no. 4, pp. 923–932, 2010.

[16] S. S. Farag and M. A. Caligiuri, "Human natural killer cell development and biology," *Blood Reviews*, vol. 20, no. 3, pp. 123–137, 2006.

[17] L. L. Lanier, "NK cell recognition," *Annual Review of Immunology*, vol. 23, pp. 225–274, 2005.

[18] Y. T. Bryceson, S. C. C. Chiang, S. Darmanin et al., "Molecular mechanisms of natural killer cell activation," *Journal of Innate Immunity*, vol. 3, no. 3, pp. 216–226, 2011.

[19] M. G. Joyce and P. D. Sun, "The structural basis of ligand recognition by natural killer cell receptors," *Journal of Biomedicine and Biotechnology*, vol. 2011, Article ID 203628, 15 pages, 2011.

[20] B. Zafirova, F. M. Wensveen, M. Gulin, and B. Polić, "Regulation of immune cell function and differentiation by the NKG2D receptor," *Cellular and Molecular Life Sciences*, vol. 68, no. 21, pp. 3519–3529, 2011.

[21] A. K. Purdy and K. S. Campbell, "Natural killer cells and cancer: regulation by the killer cell ig-like receptors (KIR)," *Cancer Biology and Therapy*, vol. 8, no. 23, pp. 2211–2220, 2009.

[22] R. A. Eagle and J. Trowsdale, "Promiscuity and the single receptor: NKG2D," *Nature Reviews Immunology*, vol. 7, no. 9, pp. 737–744, 2007.

[23] D. H. Raulet, "Roles of the NKG2D immunoreceptor and its ligands," *Nature Reviews Immunology*, vol. 3, no. 10, pp. 781–790, 2003.

[24] P. Li, S. T. Willie, S. Bauer, D. L. Morris, T. Spies, and R. K. Strong, "Crystal structure of the MHC class I homolog MIC-A, a $\gamma\delta$ T cell ligand," *Immunity*, vol. 10, no. 5, pp. 577–584, 1999.

[25] P. Li, D. L. Morris, B. E. Willcox, A. Steinle, T. Spies, and R. K. Strong, "Complex structure of the activating immunoreceptor NKG2D and its MHC class I-like ligand MICA," *Nature Immunology*, vol. 2, no. 5, pp. 443–451, 2001.

[26] M. A. Holmes, P. Li, E. W. Petersdorf, and R. K. Strong, "Structural studies of allelic diversity of the MHC class I homolog MIC-B, a stress-inducible ligand for the activating immunoreceptor NKG2D," *Journal of Immunology*, vol. 169, no. 3, pp. 1395–1400, 2002.

[27] L. Moretta, C. Bottino, D. Pende, R. Castriconi, M. C. Mingari, and A. Moretta, "Surface NK receptors and their ligands on tumor cells," *Seminars in Immunology*, vol. 18, no. 3, pp. 151–158, 2006.

[28] N. Stern-Ginossar and O. Mandelboim, "An integrated view of the regulation of NKG2D ligands," *Immunology*, vol. 128, no. 1, pp. 1–6, 2009.

[29] S. Gasser, S. Orsulic, E. J. Brown, and D. H. Raulet, "The DNA damage pathway regulates innate immune system ligands of the NKG2D receptor," *Nature*, vol. 436, no. 7054, pp. 1186–1190, 2005.

[30] K. Yamamoto, Y. Fujiyama, A. Andoh, T. Bamba, and H. Okabe, "Oxidative stress increases MICA and MICB gene expression in the human colon carcinoma cell line (CaCo-2)," *Biochimica et Biophysica Acta*, vol. 1526, no. 1, pp. 10–12, 2001.

[31] A. Frigoul and M.-P. Lefranc, "MICA: standardized IMGT allele nomenclature, polymorphisms and diseases," in *Recent Research Developments in Human Genetics*, S. G. Pandalai, Ed., vol. 3, pp. 95–145, 2005.

[32] E. W. Petersdorf, K. B. Shuler, G. M. Longton, T. Spies, and J. A. Hansen, "Population study of allelic diversity in the human MHC class I-related MIC-A gene," *Immunogenetics*, vol. 49, no. 7-8, pp. 605–612, 1999.

[33] F. Zhu, H. Zhao, Y. He et al., "Distribution of MICA diversity in the Chinese Han population by polymerase chain reaction sequence-based typing for exons 2-6," *Tissue Antigens*, vol. 73, no. 4, pp. 358–363, 2009.

[34] M. L. C. Marin, C. R. Savioli, J. H. Yamamoto, J. Kalil, and A. C. Goldberg, "MICA polymorphism in a sample of the São Paulo population, Brazil," *European Journal of Immunogenetics*, vol. 31, no. 2, pp. 63–71, 2004.

[35] X. Gao, R. M. Single, P. Karacki, D. Marti, S. J. O'Brien, and M. Carrington, "Diversity of MICA and linkage disequilibrium with HLA-B in two North American populations," *Human Immunology*, vol. 67, no. 3, pp. 152–158, 2006.

[36] D. Lucas, J. A. Campillo, R. López-Hernández et al., "Allelic diversity of MICA gene and MICA/HLA-B haplotypic variation in a population of the Murcia region in southeastern Spain," *Human Immunology*, vol. 69, no. 10, pp. 655–660, 2008.

[37] W. Tian, D. A. Boggs, G. Uko et al., "MICA, HLA-B haplotypic variation in five population groups of sub-Saharan African ancestry," *Genes and Immunity*, vol. 4, no. 7, pp. 500–505, 2003.

[38] A. Cambra, I. Muñoz-Saá, C. Crespí et al., "MICA-HLA-B haplotype diversity and linkage disequilibrium in a population of Jewish descent from Majorca (the Balearic Islands)," *Human Immunology*, vol. 70, no. 7, pp. 513–517, 2009.

[39] V. Ďurmanová, J. Tirpakova, M. Stuchlikova et al., "Characterization of MICA gene polymorphism of HLA complex in the Slovak population," *Annals of Human Biology*, vol. 38, no. 5, pp. 570–576, 2011.

[40] D. Cosman, J. Müllberg, C. L. Sutherland et al., "ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor," *Immunity*, vol. 14, no. 2, pp. 123–133, 2001.

[41] C. Bottino, R. Biassoni, R. Millo, L. Moretta, and A. Moretta, "The human natural cytotoxicity receptors (NCR) that induce HLA class I-independent NK cell triggering," *Human Immunology*, vol. 61, no. 1, pp. 1–6, 2000.

[42] M. Vitale, M. Falco, R. Castriconi et al., "Identification of NKp80, a novel triggering molecule expressed by human NK cells," *European Journal of Immunology*, vol. 31, no. 1, pp. 233–242, 2001.

[43] C. Fauriat, S. Just-Landi, F. Mallet et al., "Deficient expression of NCR in NK cells from acute myeloid leukemia: Evolution during leukemia treatment and impact of leukemia cells in NCR dull phenotype induction," *Blood*, vol. 109, no. 1, pp. 323–330, 2007.

[44] R. T. Costello, B. Knoblauch, C. Sanchez, D. Mercier, T. Le Treut, and G. Sébahoun, "Expression of natural killer cell activating receptors in patients with chronic lymphocytic leukaemia," *Immunology*, vol. 135, no. 2, pp. 151–157, 2012.

[45] A. Glasner, H. Ghadially, C. Gur et al., "Recognition and prevention of tumor metastasis by the NK receptor NKp46/NCR1," *Journal of Immunology*, vol. 188, no. 6, pp. 2509–2515, 2012.

[46] S. Morgado, B. Sanchez-Correa, J. G. Casado et al., "NK cell recognition and killing of melanoma cells is controlled by multiple activating receptor-ligand interactions," *Journal of Innate Immunity*, vol. 3, no. 4, pp. 365–373, 2011.

[47] Y. Li, Q. Wang, and R. A. Mariuzza, "Structure of the human activating natural cytotoxicity receptor NKp30 bound to its tumor cell ligand B7-H6," *Journal of Experimental Medicine*, vol. 208, no. 4, pp. 703–714, 2011.

[48] T. Kaifu, B. Escalière, L. N. Gastinel, E. Vivier, and M. Baratin, "B7-H6/NKp30 interaction: a mechanism of alerting NK cells against tumors," *Cellular and Molecular Life Sciences*, vol. 68, no. 21, pp. 3531–3539, 2011.

[49] E. Pogge von Strandmann, V. R. Simhadri, B. von Tresckow et al., "Human leukocyte antigen-B-associated transcript 3 is released from tumor vells and engages the NKp30 receptor on natural killer cells," *Immunity*, vol. 27, no. 6, pp. 965–974, 2007.

[50] M. L. Hecht, B. Rosental, T. Horlacher et al., "Natural cytotoxicity receptors NKp30, NKp44 and NKp46 bind to different heparan sulfate/heparin sequences," *Journal of Proteome Research*, vol. 8, no. 2, pp. 712–720, 2009.

[51] M. Jarahian, C. Watzl, P. Fournier et al., "Activation of natural killer cells by Newcastle disease virus hemagglutinin-neuraminidase," *Journal of Virology*, vol. 83, no. 16, pp. 8108–8121, 2009.

[52] M. Carlsten, N. K. Björkström, H. Norell et al., "DNAX accessory molecule-1 mediated recognition of freshly isolated ovarian carcinoma by resting natural killer cells," *Cancer Research*, vol. 67, no. 3, pp. 1317–1325, 2007.

[53] R. Castriconi, A. Dondero, M. V. Corrias et al., "Natural killer cell-mediated killing of freshly isolated neuroblastoma cells: Critical role of DNAX accessory molecule-1-poliovirus receptor interaction," *Cancer Research*, vol. 64, no. 24, pp. 9180–9184, 2004.

[54] C. J. Chan, D. M. Andrews, N. M. McLaughlin et al., "DNAM-1/CD155 interactions promote cytokine and NK cell-mediated suppression of poorly immunogenic melanoma metastases," *Journal of Immunology*, vol. 184, no. 2, pp. 902–911, 2010.

[55] T. Lakshmikanth, S. Burke, T. H. Ali et al., "NCRs and DNAM-1 mediate NK cell recognition and lysis of human and mouse melanoma cell lines in vitro and in vivo," *Journal of Clinical Investigation*, vol. 119, no. 5, pp. 1251–1263, 2009.

[56] Z. Zhang, T. Su, L. He et al., "Identification and functional analysis of ligands for natural killer cell activating receptors in colon carcinoma," *The Tohoku journal of experimental medicine*, vol. 226, no. 1, pp. 59–68, 2012.

[57] Z. Huang, B. Fu, S. G. Zheng et al., "Involvement of $CD226^+$ NK cells in immunopathogenesis of systemic lupus erythematosus," *Journal of Immunology*, vol. 186, no. 6, pp. 3421–3431, 2011.

[58] A. K. Maiti, X. Kim-Howard, P. Viswanathan et al., "Non-synonymous variant (Gly307Ser) in CD226 is associated with susceptibility to multiple autoimmune diseases," *Rheumatology*, vol. 49, no. 7, Article ID kep470, pp. 1239–1244, 2010.

[59] J. M. L. Tjon, Y. M. C. Kooy-Winkelaar, G. J. Tack et al., "DNAM-1 mediates epithelial cell-specific cytotoxicity of aberrant intraepithelial lymphocyte lines from refractory celiac disease type II patients," *Journal of Immunology*, vol. 186, no. 11, pp. 6304–6312, 2011.

[60] N. Wagtmann, S. Rajagopalan, C. C. Winter, M. Peruzzi, and E. O. Long, "Killer cell inhibitory receptors specific for HLA-C and HLA-B identified by direct binding and by functional transfer," *Immunity*, vol. 3, no. 6, pp. 801–809, 1995.

[61] V. M. Braud, D. S. J. Allan, C. A. O'Callaghan et al., "HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C," *Nature*, vol. 391, no. 6669, pp. 795–799, 1998.

[62] F. Borrego, M. Ulbrecht, E. H. Weiss, J. E. Coligan, and A. G. Brooks, "Recognition of human histocompatibility leukocyte antigen (HLA)-E complexed with HLA class I signal sequence-derived peptides by CD94/NKG2 confers protection from natural killer cell-mediated lysis," *Journal of Experimental Medicine*, vol. 187, no. 5, pp. 813–818, 1998.

[63] K. Karre, H. G. Ljunggren, G. Piontek, and R. Kiessling, "Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy," *Nature*, vol. 319, no. 6055, pp. 675–678, 1986.

[64] B. Huard and K. Früh, "A role for MHC class I down-regulation in NK cell lysis of herpes virus-infected cells," *European Journal of Immunology*, vol. 30, no. 2, pp. 509–515, 2000.

[65] J. Yu, G. Heller, J. Chewning, S. Kim, W. M. Yokoyama, and K. C. Hsu, "Hierarchy of the human natural killer cell response is determined by class and quantity of inhibitory receptors for self-HLA-B and HLA-C ligands," *Journal of Immunology*, vol. 179, no. 9, pp. 5977–5989, 2007.

[66] J. Van Bergen, A. Thompson, C. Retière, J. Trowsdale, and F. Koning, "Cutting edge: killer Ig-like receptors mediate

"missing self" recognition in vivo," *Journal of Immunology*, vol. 182, no. 5, pp. 2569–2572, 2009.

[67] A. Lin, H. Xu, and W. Yan, "Modulation of HLA expression in human cytomegalovirus immune evasion," *Cellular & Molecular Immunology*, vol. 4, no. 2, pp. 91–98, 2007.

[68] N. J. Chalupny, A. Rein-Weston, S. Dosch, and D. Cosman, "Down-regulation of the NKG2D ligand MICA by the human cytomegalovirus glycoprotein UL142," *Biochemical and Biophysical Research Communications*, vol. 346, no. 1, pp. 175–181, 2006.

[69] O. Ashiru, N. J. Bennett, L. H. Boyle, M. Thomas, J. Trowsdale, and M. R. Wills, "NKG2D ligand MICA is retained in the cis-Golgi apparatus by human cytomegalovirus protein UL142," *Journal of Virology*, vol. 83, no. 23, pp. 12345–12354, 2009.

[70] M. Thomas, M. Wills, and P. J. Lehner, "Natural killer cell evasion by an E3 ubiquitin ligase from Kaposi's sarcoma-associated herpesvirus," *Biochemical Society Transactions*, vol. 36, no. 3, pp. 459–463, 2008.

[71] M. Thomas, J. M. Boname, S. Field et al., "Down-regulation of NKG2D and NKp80 ligands by Kaposi's sarcoma-associated herpesvirus K5 protects against NK cell cytotoxicity," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 5, pp. 1656–1661, 2008.

[72] D. Schepis, M. D'Amato, M. Studahl, T. Bergström, K. Kärre, and L. Berg, "Herpes simplex virus infection downmodulates NKG2D ligand expression," *Scandinavian Journal of Immunology*, vol. 69, no. 5, pp. 429–436, 2009.

[73] S. E. Chisholm, K. Howard, M. V. Gómez, and H. T. Reyburn, "Expression of ICP0 is sufficient to trigger natural killer cell recognition of herpes simplex virus-infected cells by natural cytotoxicity receptors," *Journal of Infectious Diseases*, vol. 195, no. 8, pp. 1160–1168, 2007.

[74] C. L. Schneider and A. W. Hudson, "The human herpesvirus-7 (HHV-7) U21 immunoevasin subverts NK-mediated cytoxicity through modulation of MICA and MICB," *PLoS Pathogens*, vol. 7, no. 11, Article ID e1002362, 2011.

[75] S. A. Welte, C. Sinzger, S. Z. Lutz et al., "Selective intracellular retention of virally induced NKG2D ligands by the human cytomegalovirus UL16 glycoprotein," *European Journal of Immunology*, vol. 33, no. 1, pp. 194–203, 2003.

[76] J. Wu, N. J. Chalupny, T. J. Manley, S. R. Riddell, D. Cosman, and T. Spies, "Intracellular retention of the MHC class I-related chain B ligand of NKG2D by the human cytomegalovirus UL16 glycoprotein," *Journal of Immunology*, vol. 170, no. 8, pp. 4196–4200, 2003.

[77] C. Dunn, N. J. Chalupny, C. L. Sutherland et al., "Human cytomegalovirus glycoprotein UL16 causes intracellular sequestration of NKG2D ligands, protecting against natural killer cell cytotoxicity," *Journal of Experimental Medicine*, vol. 197, no. 11, pp. 1427–1439, 2003.

[78] N. Stern-Ginossar, N. Elefant, A. Zimmermann et al., "Host immune system gene targeting by a viral miRNA," *Science*, vol. 317, no. 5836, pp. 376–381, 2007.

[79] D. Nachmani, N. Stern-Ginossar, R. Sarid, and O. Mandelboim, "Diverse herpesvirus microRNAs target the stress-induced immune ligand MICB to escape recognition by natural killer cells," *Cell Host and Microbe*, vol. 5, no. 4, pp. 376–385, 2009.

[80] H. T. Reyburn, O. Mandelboim, M. Valés-Gómez, D. M. Davis, L. Pazmany, and J. L. Strominger, "The class I MHC homologue of human cytomegalovirus inhibits attack by natural killer cells," *Nature*, vol. 386, no. 6624, pp. 514–517, 1997.

[81] Z. Yang and P. J. Bjorkman, "Structure of UL18, a peptide-binding viral MHC mimic, bound to a host inhibitory receptor," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 29, pp. 10095–10100, 2008.

[82] P. Tomasec, "Surface expression of HLA-E, an inhibitor of natural killer cells, enhanced by human cytomegalovirus gpUL40," *Science*, vol. 287, no. 5455, pp. 1031–1033, 2000.

[83] M. Ulbrecht, S. Martinozzi, M. Grzeschik et al., "Cutting edge: the human cytomegalovirus UL40 gene product contains a ligand for HLA-E and prevents NK cell-mediated lysis," *Journal of Immunology*, vol. 164, no. 10, pp. 5019–5022, 2000.

[84] E. C. Y. Wang, B. McSharry, C. Retiere et al., "UL40-mediated NK evasion during productive infection with human cytomegalovirus," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 11, pp. 7570–7575, 2002.

[85] S. Ishido, C. Wang, B. S. Lee, G. B. Cohen, and J. U. Jung, "Downregulation of major histocompatibility complex class I molecules by Kaposi's sarcoma-associated herpesvirus K3 and K5 proteins," *Journal of Virology*, vol. 74, no. 11, pp. 5300–5309, 2000.

[86] M. Haque, K. Ueda, K. Nakano et al., "Major histocompatibility complex class I molecules are down-regulated at the cell surface by the K5 protein encoded by Kaposi's sarcoma-associated herpesvirus/human herpesvirus-8," *Journal of General Virology*, vol. 82, part 5, pp. 1175–1180, 2001.

[87] E. Bartee, M. Mansouri, B. T. H. Nerenberg, K. Gouveia, and K. Früh, "Downregulation of major histocompatibility complex class I by human ubiquitin ligases related to viral Immune evasion proteins," *Journal of Virology*, vol. 78, no. 3, pp. 1109–1120, 2004.

[88] A. B. Hill, B. C. Barnett, A. J. McMichael, and D. J. McGeoch, "HLA class I molecules are not transported to the cell surface in cells infected with herpes simplex virus types 1 and 2," *Journal of Immunology*, vol. 152, no. 6, pp. 2736–2741, 1994.

[89] A. Hill, P. Juovic, I. York et al., "Herpes simplex virus turns off the TAP to evade host immunity," *Nature*, vol. 375, no. 6530, pp. 411–415, 1995.

[90] I. A. York, C. Roop, D. W. Andrews, S. R. Riddell, F. L. Graham, and D. C. Johnson, "A cytosolic herpes simplex virus protein inhibits antigen presentation to $CD8^+$ T lymphocytes," *Cell*, vol. 77, no. 4, pp. 525–535, 1994.

[91] S.-H. Sin and D. P. Dittmer, "Cytokine homologs of human gammaherpesviruses," *Journal of Interferon and Cytokine Research*, vol. 32, no. 2, pp. 53–59, 2012.

[92] N. P. Croft, C. Shannon-Lowe, A. I. Bell et al., "Stage-specific inhibition of MHC class I presentation by the epstein-barr virus BNLF2a protein during virus lytic cycle," *PLoS Pathogens*, vol. 5, no. 6, Article ID e1000490, 2009.

[93] D. Horst, V. Favaloro, F. Vilardi et al., "EBV protein BNLF2a exploits host tail-anchored protein integration machinery to inhibit TAP," *Journal of Immunology*, vol. 186, no. 6, pp. 3594–3605, 2011.

[94] J. Zuo, L. L. Quinn, J. Tamblyn et al., "The Epstein-Barr virus-encoded BILF1 protein modulates immune recognition of endogenously processed antigen by targeting major histocompatibility complex class I molecules trafficking on both the exocytic and endocytic pathways," *Journal of Virology*, vol. 85, no. 4, pp. 1604–1614, 2011.

[95] N. L. Glosson and A. W. Hudson, "Human herpesvirus-6A and -6B encode viral immunoevasins that downregulate class I MHC molecules," *Virology*, vol. 365, no. 1, pp. 125–135, 2007.

[96] N. L. Glosson, P. Gonyo, N. A. May et al., "Insight into the mechanism of human herpesvirus 7 U21-mediated diversion of class I MHC molecules to lysosomes," *Journal of Biological Chemistry*, vol. 285, no. 47, pp. 37016–37029, 2010.

[97] N. A. May, N. L. Glosson, and A. W. Hudson, "Human herpesvirus 7 U21 downregulates classical and nonclassical class I major histocompatibility complex molecules from the cell surface," *Journal of Virology*, vol. 84, no. 8, pp. 3738–3751, 2010.

[98] A. W. Hudson, D. Blom, P. M. Howley, and H. L. Ploegh, "The ER-lumenal domain of the HHV-7 immunoevasin U21 directs class I MHC molecules to lysosomes," *Traffic*, vol. 4, no. 12, pp. 824–837, 2003.

[99] A. Abendroth, I. Lin, B. Slobedman, H. Ploegh, and A. M. Arvin, "Varicella-zoster virus retains major histocompatibility complex class I proteins in the golgi compartment of infected cells," *Journal of Virology*, vol. 75, no. 10, pp. 4878–4888, 2001.

[100] A. J. Eisfeld, M. B. Yee, A. Erazo, A. Abendroth, and P. R. Kinchington, "Downregulation of class I major histocompatibility complex surface expression by varicella-zoster virus involves open reading frame 66 protein kinase-dependent and -independent mechanisms," *Journal of Virology*, vol. 81, no. 17, pp. 9034–9049, 2007.

[101] C. C. Leong, T. L. Chapman, P. J. Bjorkman et al., "Modulation of natural killer cell cytotoxicity in human cytomegalovirus infection: The role of endogenous class I major histocompatibility complex and a viral class I homolog," *Journal of Experimental Medicine*, vol. 187, no. 10, pp. 1681–1687, 1998.

[102] D. Cosman, N. Fanger, L. Borges et al., "A novel immunoglobulin superfamily receptor for cellular and viral MHC class I molecules," *Immunity*, vol. 7, no. 2, pp. 273–282, 1997.

[103] M. R. Wills, O. Ashiru, M. B. Reeves et al., "Human cytomegalovirus encodes an MHC Class I-like molecule (UL142) that functions to inhibit NK cell lysis," *Journal of Immunology*, vol. 175, no. 11, pp. 7457–7465, 2005.

[104] M. Llano, M. Gumá, M. Ortega, A. Angulo, and M. López-Botet, "Differential effects of US2, US6, and US11 human cytomegalovirus proteins of HLA class Ia and HLA-E expression: Impact on target susceptibility to NK cell subsets," *European Journal of Immunology*, vol. 33, no. 10, pp. 2744–2754, 2003.

[105] M. Wittenbrink, J. Spreu, and A. Steinle, "Differential NKG2D binding to highly related human NKG2D ligands ULBP2 and RAET1G is determined by a single amino acid in the α2 domain," *European Journal of Immunology*, vol. 39, no. 6, pp. 1642–1651, 2009.

[106] S. Müller, G. Zocher, A. Steinle, and T. Stehle, "Structure of the HCMV UL16-MICB complex elucidates select binding of a viral immunoevasin to diverse NKG2D ligands," *PLoS Pathogens*, vol. 6, no. 1, Article ID e1000723, 2010.

[107] M. Colonna, S. Jonjic, and C. Watzl, "Natural killer cells: fighting viruses andmuch more," *Nature Immunology*, vol. 12, no. 2, pp. 107–110, 2011.

[108] E. S. Mocarski, "Immune escape and exploitation strategies of cytomegaloviruses: impact on and imitation of the major histocompatibility system," *Cellular Microbiology*, vol. 6, no. 8, pp. 707–717, 2004.

[109] S. Ishido, J. K. Choi, B. S. Lee et al., "Inhibition of natural killer cell-mediated cytotoxicity by Kaposi's sarcoma-associated herpesvirus K5 protein," *Immunity*, vol. 13, no. 3, pp. 365–374, 2000.

[110] S. Dupuy, M. Lambert, D. Zucman et al., "Human herpesvirus 8 (HHV8) sequentially shapes the NK cell repertoire during the course of asymptomatic infection and Kaposi sarcoma," *PLoS Pathogens*, vol. 8, no. 1, Article ID e1002486, 2012.

[111] R. E. Means, J. K. Choi, H. Nakamura, Y. H. Chung, S. Ishido, and J. U. Jung, "Immune evasion strategies of Kaposi's sarcoma-associated herpesvirus," *Current Topics in Microbiology and Immunology*, vol. 269, pp. 187–201, 2002.

[112] R. Tomazin, N. E. G. Van Schoot, K. Goldsmith et al., "Herpes simplex virus type 2 ICP47 inhibits human TAP but not mouse tap," *Journal of Virology*, vol. 72, no. 3, pp. 2560–2563, 1998.

[113] M. Lafon, C. Prehaud, F. Megret et al., "Modulation of HLA-G expression in human neural cells after neurotropic viral infections," *Journal of Virology*, vol. 79, no. 24, pp. 15226–15237, 2005.

[114] D. S. Kaufman, R. A. Schoon, and P. J. Leibson, "Role for major histocompatibility complex class I in regulating natural killer cell-mediated killing of virus-infected cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 89, no. 17, pp. 8337–8341, 1992.

[115] I. Y. Pappworth, E. C. Wang, and M. Rowe, "The switch from latent to productive infection in Epstein-Barr virus-infected B cells is associated with sensitization to NK cell killing," *Journal of Virology*, vol. 81, no. 2, pp. 474–482, 2007.

[116] H. Song, H. Park, J. Kim et al., "IDO metabolite produced by EBV-transformed B cells inhibits surface expression of NKG2D in NK cells via the c-Jun N-terminal kinase (JNK) pathway," *Immunology Letters*, vol. 136, no. 2, pp. 187–193, 2011.

[117] A. Sawada, E. Sato, M. Koyama et al., "NK-cell repertoire is feasible for diagnosing Epstein-Barr virus-infected NK-cell lymphoproliferative disease and evaluating the treatment effect," *American Journal of Hematology*, vol. 81, no. 8, pp. 576–581, 2006.

[118] K. Kida, R. Isozumi, and M. Ito, "Killing of human herpes virus 6-infected cells by lymphocytes cultured with interleukin-2 or -12," *Pediatrics International*, vol. 42, no. 6, pp. 631–636, 2000.

[119] L. Flamand, I. Stefanescu, and J. Menezes, "Human herpesvirus-6 enhances natural killer cell cytotoxicity via IL-15," *Journal of Clinical Investigation*, vol. 97, no. 6, pp. 1373–1381, 1996.

[120] B. N. Atedzoe, A. Ahmad, and J. Menezes, "Enhancement of natural killer cell cytotoxicity by the human herpesvirus-7 via IL-15 induction," *Journal of Immunology*, vol. 159, no. 10, pp. 4966–4972, 1997.

[121] A. B. Tilden, R. Cauda, and C. E. Grossi, "Demonstration of NK cell-mediated lysis of varicella-zoster virus (VZV)-infected cells: characterization of the effector cells," *Journal of Immunology*, vol. 136, no. 11, pp. 4243–4248, 1986.

[122] M. Ito, S. Bandyopadhyay, and M. Matsumoto-Kobayashi, "Interleukin 2 enhances natural killing of varicella-zoster virus-infected targets," *Clinical and Experimental Immunology*, vol. 65, no. 1, pp. 182–189, 1986.

[123] A. R. Hayward, M. Herberger, and M. Lazslo, "Cellular interactions in the lysis of varicella-zoster virus infected human fibroblasts," *Clinical and Experimental Immunology*, vol. 63, no. 1, pp. 141–146, 1986.

[124] F. Rozenberg, C. Deback, and H. Agut, "Herpes simplex encephalitis: from virus to therapy," *Infectious Disorders Drug Targets*, vol. 11, no. 3, pp. 235–250, 2011.

[125] J.-P. Stahl, A. Mailles, L. Dacheux, and P. Morand, "Epidemiology of viral encephalitis in 2011," *Medecine et Maladies Infectieuses*, vol. 41, no. 9, pp. 453–464, 2011.

[126] S. Gantt and C. Casper, "Human herpesvirus 8-associated neoplasms: the roles of viral replication and antiviral treatment," *Current Opinion in Infectious Diseases*, vol. 24, no. 4, pp. 295–301, 2011.

[127] G. S. Taylor and D. J. Blackbourn, "Infectious agents in human cancers: lessons in immunity and immunomodulation from gammaherpesviruses EBV and KSHV," *Cancer Letters*, vol. 305, no. 2, pp. 263–278, 2011.

[128] C. Chisholm and L. Lopez, "Cutaneous infections caused by herpesviridae: a review," *Archives of Pathology and Laboratory Medicine*, vol. 135, no. 10, pp. 1357–1362, 2011.

[129] L. J. Al-Dujaili, P. P. Clerkin, C. Clement et al., "Ocular herpes simplex virus: how are latency, reactivation, recurrent disease and therapy interrelated?" *Future Microbiology*, vol. 6, no. 8, pp. 877–907, 2011.

[130] A. Berardi, L. Lugli, C. Rossi et al., "Neonatal herpes simplex virus," *Journal of Maternal-Fetal and Neonatal Medicine*, vol. 24, supplement 1, pp. 88–90, 2011.

[131] G. Fulci, L. Breymann, D. Gianni et al., "Cyclophosphamide enhances glioma virotherapy by inhibiting innate immune responses," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 34, pp. 12873–12878, 2006.

[132] G. Fulci, N. Dmitrieva, D. Gianni et al., "Depletion of peripheral macrophages and brain microglia increases brain tumor titers of oncolytic viruses," *Cancer Research*, vol. 67, no. 19, pp. 9398–9406, 2007.

[133] H. Wakimoto, G. Fulci, E. Tyminski, and E. A. Chiocca, "Altered expression of antiviral cytokine mRNAs associated with cyclophosphamide's enhancement of viral oncolysis," *Gene Therapy*, vol. 11, no. 2, pp. 214–223, 2004.

[134] W. Haedicke, F. C. S. Ho, A. Chott et al., "Expression of CD94/NKG2A and killer immunoglobulin-like receptors in NK cells and a subset of extranodal cytotoxic T-cell lymphomas," *Blood*, vol. 95, no. 11, pp. 3628–3630, 2000.