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Interferon, the Cell Cycle and Herpesvirus

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1. Introduction

Herpesviruses are a large group of successful, and widely distributed, double-stranded DNA viruses of serious medical and veterinary importance. Although they infect many different animal species, they are host specific at the individual species level. On the other hand, they share a common life style, first with an acute infection in epithelial cells which is followed by the establishment of persistence in neurons (α-herpesvirus), monocytes (β-herpesvirus), or B lymphocytes (γ-herpesvirus). The varied pathology of these different groups of herpesvirus is typically associated with reactivation of a persistent infection and the subsequent production of virus. Thus, the immune system faces three distinct challenges: how to control the acute phase, the persistent virus, and the consequences of reactivation. For this reason, control of herpesvirus infection calls on the many functional arms of both the innate and adaptive immune systems, which in turn have exerted the selection pressure that has driven the evolution of many strategies of immune evasion. This chapter will focus on herpesviruses host evasion genes manipulating cell cycle progression and interferon.

All members of order *Herpesvirales* have a biphasic infection cycle consisting of replicative (lytic) and latent phases. During the lytic cycle and viral reactivation, most of the viral genes are expressed in a cascade manner and large numbers of infectious virus particles are released. Latency, on the other hand, is characterized by limited gene expression, lack of virion production and, in the case of γ-herpesviruses, is associated with immortalization and transformation of infected cells. Virus survival at each phase depends on evasion of the host immune response. Thus, the escape from immune detection in the early phases of infection may be almost as important as in the latent phase (Vider-Shalit *et al.*, 2007). The typical herpesvirus life cycle is a challenge for the development of a global antiviral therapy or protective vaccines. Although all herpesviruses present a similar lytic phase and are able to establish latency in a specific set of cells, the cell types in which they remain latent, and thus have evolved virus host cell evasion molecular mechanisms, differ widely from one virus to another (Pellet & Roizman, 2007). One promising approach is to explore new viral targets, particularly viral proteins involved in host immune evasion. An effective herpesvirus vaccine would therefore be a genetically targeted mutant with one or more non-immunogenic host evasion genes deleted, and with appropriate investigation of the pathogenesis to ensure safety.

Bioinformatic analysis of putative homologues showed that 39 conserved herpesvirus protein families and 20 single proteins had significant sequence similarity to human gene products,

with 54% of them being involved in host-virus interaction, particularly control of apoptosis and immune response (Holzerlandt *et al.*, 2002). There are, however, evasion proteins encoded by genes without sequence homology to cellular genes. In these cases, the viral protein function can only be accessed by functional assays or sophisticated structural assays, such as x-ray crystallography (Cooray *et al.*, 2007). Virus proteins without sequence homology with cellular genes can still be functional homologues of cellular proteins. One interesting example is the HSV-1 US3, a viral protein kinase, that has no sequence homology to the cellular kinase Akt, yet it is able to phosphorylate tuberous sclerosis complex 2 (TSC2) on S939 and T1462, the same sites targeted by cellular Akt to inhibit TSC activity and activate mTORC1 in uninfected cells. This strategy allows the virus to bypass the strict limits normally imposed on the cellular Akt, promoting mTORC1 activation even when Akt activity is low or undetectable, as may be the case in non-proliferating cells (Chuluunbaatar *et al.*, 2010).

Virus host evasion strategies conserved in all herpesviruses are likely to manipulate conserved cellular pathways that are regulated by all herpesviruses. Others, on the other hand, may be restricted to one subfamily or species, with a function related to a more restricted specific aspect of the virus life cycle, particularly during latency. While the latter can be explored for the development of a specific herpesvirus therapy, functional studies of herpesvirus homologous protein families are advantageous for a global herpesvirus treatment.

Herpesviruses have evolved a wide repertoire of host evasion genes that impact on many components of the immune response, such as antigen presentation, autophagy, and apoptosis, which have been extensively reviewed and are not included in this chapter. Here, we focus on two virus proteins conserved in all herpesviruses, ORF36 and UL24. The first contains a conserved kinase domain with cellular homology, while UL24 is an unassigned gene with no cellular homolog. Their roles in manipulating interferon and the cell cycle have been the focus of our laboratory and will be described in detail.

2. The immune response to viruses

The immune system is an astoundingly resourceful defence system which has evolved to protect animals from external and internal threats, that is, invading pathogens and tissue damage, respectively. It is able to generate a number of different cells, secreted effector molecules and intracellular mechanisms that act independently and together. This wide variety of possible responses reflects the equally wide variety of extracellular and intracellular threats and life-styles. As a necessary correlate, the immune system must distinguish between pathogen molecules ("non self") and its own cells and proteins ("self"), and also select and apply the most "appropriate" immune effector mechanism.

Following the elimination of the pathogen, it is equally important to switch off the selected immune effector mechanisms. Failure at either the level of self-non-self discrimination, or appropriate regulation of immune responses, can lead to non-infectious diseases - for example, autoimmune and inflammatory diseases. As pathogens have evolved many mechanisms to manipulate the immune system, however, these provide "ready-made tools" for the development of novel therapeutic approaches; for example, viruses have evolved a variety of mechanisms to inhibit the inflammatory response.

The immune response against virus infections can be divided into innate and adaptive defence components. The innate immune response is the first line of defence as it is always present and rapidly activated in a normal host upon exposure to the invading virus. Many viral infections are resolved by the innate immune system before intervention by the

adaptive immune system, which is the second-line of defence to be mobilized. This adaptive response comprises the antibody response and the lymphocyte cell-mediated response, usually called humoral and cell-mediated immune responses, respectively (Flint, 2004). A successful immune response to an infection must be appropriately selected and regulated, as inefficient or inappropriate regulation can fail to eliminate the invader and/or cause disease. Viruses, in particular, have evolved multiple strategies to down-regulate, or terminate, immune responses, and thus provide enormous potential as source of strategies for immunomodulation, as indicated above.

This section will focus on virus manipulation of innate immunity, particularly the interferon system.

2.1 Innate immunity

Innate immunity is the most immediate line of response to pathogens, which needs to be potent and rapid, and functions to eliminate and prevent the spread of the pathogen prior to the subsequent action of the adaptive immune response. However, it must also be transient because its continued activity can damage the host. Importantly, innate immunity also shapes and regulates the subsequent adaptive immune response as a result of the combined influence of antigen presentation and the secreted cytokine/chemokine profile. Among these secreted effectors, interferon is absolutely essential for virus immunity.

Viruses are recognised as foreign, and thus potentially dangerous, by a limited number of germline-encoded host pattern-recognition receptors (PRRs), which recognize viral pathogen-associated molecular patterns (PAMPs). As PAMPS are highly conserved structures, the corresponding PRRs are also conserved. Of particular interest for virus infection is the TLR3 molecule that interacts with double-stranded RNA of viral origin. There is, in addition, TLR7/8 and TLR9 which also localise to endosomes and recognise viral DNA. Cells also recognise viruses in their cytoplasm through the retinoic acid-inducible gene I (RIG-I)-like helicases (RLHs), consisting of RIG-I and the melanoma differentiation-associated gene-5 (MDA5), that recognise viral RNA. Other sensors such as DAI or IFI16 detect viral DNA. These molecules initiate similar signalling transduction pathways which also results in the production of type I IFN and pro-inflammatory cytokines (Takeuchi & Akira, 2007). Pro-inflammatory cytokines can also be induced independently of IFN, by activation of the inflammasome which also has specific sensors localised both in endosomes and in the cytoplasm.

2.2 The interferon system

The interferons constitute three groups of cytokines (type I, II, and III) with overlapping, specific and redundant activities, and plays a major role in virus immunity. They are secreted by a variety of cell types as a result of disparate pathogen molecular signals, which in turn activate similarly overlapping, specific, and redundant intracellular signalling pathways. The complexity of the system has presumably evolved together with the co-evolution of virus mechanisms for its subversion. Thus, in order to discuss virus strategies for the manipulation of the interferon system, we must first describe its essentials in some detail.

Interferons control a variety of biological functions, including modulation of the immune system, regulation of apoptosis, inhibition of proliferation, induction of differentiation, and inhibition of angiogenesis. The importance of the interferon response against viral infections has been dramatised by demonstrating increased susceptibility to virus infection

of mice deficient for different components of the IFN system (Arnheiter *et al.*, 1996; Chee *et al.*, 2003; Haller *et al.*, 1981; Hefti *et al.*, 1999).

2.2.1 Induction of IFN expression

Four major families of PRRs with relevance for IFN have been identified: Toll-like receptors (TLRs), RIG-I-like receptors, NOD-like receptors, and C-type lectin receptors. Recently, several cytosolic nucleic acid sensors have also been found, such as DAI, AIM-2, RNA polymerase III and LRRFIP1. The PRRs detect pathogens invading from different routes through their differential localization in distinct subcellular compartments, such as the cell surface, endosome, and cytoplasm. Upon recognition of the pathogen, PRRs trigger major downstream signalling pathways, involving NF-κB, MAPK, and/or IRF3/7, to induce the production of inflammatory cytokines and/or type I interferons (IFNs), thereby leading to antimicrobial immune responses.

2.2.2 Transcriptional control of IFN expression

Although, as described above, there are different routes to initiate transcription of IFN type I, the downstream kinases and transcription factors are common to all. The induction of Type I IFN is primarily regulated at the level of transcription, with the IFN regulatory factors (IRFs) IRF-1, IRF-3, IRF-5 and IRF-7 and NF-κB having major roles (Barnes *et al.*, 2001; Watanabe *et al.*, 1991; Wathelet *et al.*, 1998).

Transcription of the IFN-β gene involves the formation of a large, multi-subunit complex called the "enhanceosome". It comprises the promoter-specific transcription factors, associated structural elements, and basal transcriptional machinery to enhance gene expression. The promoter enhancer region is composed of four positive regulatory regions (PRDI-IV) (Hiscott *et al.*, 2006). The PRDI and PRDIII sequences contain sites for binding of IRF-3 and IRF-7, the PRDII site binds NF-κB heterodimers, while PRDIV binds ATF-2 and c-Jun heterodimers. Upon binding to the promoter region by the different activated transcription factors, and the high-mobility group (HMG) chromatin-associated protein HMGI(Y), the complete transcriptional machinery of the enhanceosome is formed by the additional recruitment of CBP/p300 (Honda *et al.*, 2006).

The induction of IFN-α expression is less well understood. Its promoter region contains binding sites for IRFs but lacks binding sites for NF-κB. Although the identity of the IRF member that stimulates IFN-α is uncertain, there is some evidence that IRF-7 is required for induction; for example, in fibroblast cells there is no primary induction of IFN-α gene, as IRF-7 gene expression is dependent on feedback induction by IFN-β. On the other hand, with regards to plasmocytoid DCs, which constitutively express IRF-7 and induce the expression of massive amounts of IFN type I, the induction of IFN-α is not dependent on the primary induction of IFN-β and its feedback loop (Lin *et al.*, 2000; Marie *et al.*, 1998).

The induction of type II interferon secretion is restricted to a small group of cells, with NK cells and CD8 T cells being the main source of IFN-γ. However, other cell types, such as macrophages and DCs, have also been reported to produce type II IFN under specific conditions (Darwich *et al.*, 2009).

2.2.3 Signalling responses to IFN

As secreted factors, type I IFNs regulate a range of immune responses through binding to the type I IFN receptor, composed of two subunits, IFN-α receptor 1 (IFNAR1) and IFNAR2.

Upon interferon binding, the two subunits of the receptor associate and facilitate the activation of Tyk2 and Jak1. The phosphorylation of the IFNAR1 by Tyk2 creates a docking site for STAT2 and its subsequent phosphorylation by Tyk2, while Jak1 phosphorylates STAT1 (Colamonici *et al.*, 1994; Novick *et al.*, 1994; Shuai *et al.*, 1993). The activated STATs dissociate from the receptor forming a stable heterodimer and associate with p48 (also known as IRF-9), forming the ISGF3 multimeric complex that translocates to the nucleus and binds to IFN-stimulated response elements (ISRE) present in the promoter region of IFN-stimulated genes (ISGs) (Mogensen *et al.*, 1999; Stark *et al.*, 1998). Until recently, the assembly of the ISGF3 complex was thought to be in the nucleus. However it has been recently shown that it is IFNAR2 that forms a docking site for p48 which, together with STAT1 and STAT2, then becomes acetylated (Tang *et al.*, 2007). The transcriptional co-factor CREB-binding protein (CBP) is a mediator for these acetylation reactions, implying that acetylation plays a major role in the signal transduction pathway activated by the receptors (Tang *et al.*, 2007).

The Type II IFN receptor also consists of two subunits: the IFNGR1 that associates with Jak1, and the IFNGR2, which constitutively associates with Jak2. Binding of IFN-γ to the receptor leads to its dimerization, which brings Jak1 and Jak2 into close proximity, resulting in the activation of Jak2 and trans-phosphorylation of Jak1. Activated Jaks phosphorylate the C-terminus of IFNGR1 which creates a pair of binding sites for STAT1, which are then phosphorylated and dissociate from the receptor. The STAT1 homodimer translocates to the nucleus and binds to unique elements of IFN-γ stimulated genes, the gamma-activation sequence (GAS), and stimulates transcription. Of note is the fact that type I IFN stimulation is also able to form STAT1-homodimers and leads to the induction of genes containing GAS elements in their promoter region (Bach *et al.*, 1997; Stark *et al.*, 1998). The Jak-Stat pathway plays an important role in the response to IFN and in mounting an effective and rapid anti-viral response through the induction of ISGs. The subsequent decay of the response requires negative regulators of STAT signalling, which include cytoplasmic tyrosine phosphatases, nuclear and cytoplasmic regulators and truncated forms of STAT proteins.

2.2.4 IFN-induced antiviral state

One of the major functions of interferon is the induction of an anti-viral state in cells infected by viruses. The anti-viral state is characterized by the expression of genes that are induced by interferon in order to limit virus replication and subsequent spread to neighbouring cells. The interferon stimulated genes (ISGs) are crucial components of the interferon responses as they set up the antiviral, antiproliferative and immunoregulatory state in the host cells. The best-characterized IFN inducible components that have been already reviewed are the enzymes dsRNA-dependent protein kinase (PKR), 2′,5′-oligoadenylate synthetase (2′5′OAS), and Mx proteins (Garcia *et al.*, 2006; Haller *et al.*, 2007; Silverman, 2007). Other proteins that are induced and play important roles in the antiviral response are ISG15, ISG54 and ISG56, ISG20, PML, and TRIM.

2.3 Viral evasion of interferon responses

Whatever its lifestyle, a virus will always have appropriate and complementary strategies for evasion of host defences. The interferon system is a powerful and first line of defence against virus infections, and so it is not surprising that viruses have evolved multiple means of down-regulating IFN responses. These include inhibiting IFN production, inhibiting the

IFN-mediated signalling pathways, and blocking the action of IFN-induced enzymes with antiviral activity. Even within one of these strategies, viruses have evolved multiple molecular mechanisms to achieve the same result. During the past few years, much has been learned about the molecular mechanisms used by viruses to manipulate and escape the host interferon response. The exact strategy exploited by a virus will presumably depend on the biology of the host-virus interaction, and will be a major factor that will influence the pathogenesis of that virus infection (Randall & Goodbourn, 2008).

2.4 Herpesviruses evasion of the interferon system

As herpesviruses are known to trigger the induction of type I IFN during the primary infection of a cell (Ankel *et al.*, 1998; Boehme *et al.*, 2004; Mossman *et al.*, 2001), it is not surprising that an effective evasion of these initial type I IFN responses is essential for virus replication and establishment of latency.

The modulation of interferon responses by herpesviruses is already an extensive area of research, with several proteins already been described to inhibit IFN signalling, antagonizing IFN-initiated gene transcription, and target IRF-3 and IRF-7 activation. The multiplicity of these genes reflects the importance of diminishing IFN responses for virus survival. Understanding their strategies of evasion might lead to the development of new treatments or prevention strategies for diseases associated with these viruses.

The Kaposi's Sarcoma Herpesvirus (KSHV) is one of the examples of a herpesvirus modulating interferon responses. During primary infection, a decreased transcription of type I IFN genes and subsequent binding to the receptors has been demonstrated (Naranatt *et al.*, 2004). One of the candidate genes responsible for this evasion strategy is ORF45, which is a major component of the KSHV viral tegument, thus delivered into the host cells at the most early stages of infection (Zhu *et al.*, 2005; Zhu & Yuan, 2003). In addition, ORF45 has been found to inhibit the phosphorylation and nuclear translocation of IRF-7 (Zhu *et al.*, 2002). The combination of these two effects points to the ORF45 protein as a significant contributor in the antagonism of type I IFN in *de novo* KSHV infections.

During lytic reactivation, several KSHV proteins are also involved in evasion of IFN responses. The ORF10 (RIF) inhibitory function is not at the level of IFN induction but rather at the IFN signalling pathway, by forming complexes with several critical factors of the signalling pathway such as the type I IFN receptor subunits, the janus kinases and STAT2 (Bisson *et al.*, 2009). The multiple targeting of components of this signalling pathway ensures that the ISGF3 complex is not formed and does not translocate into the nucleus thus inhibiting the transcription of ISGs. The ORF50 protein functions as a transcription factor and is essential for KSHV reactivation from latency (Sun *et al.*, 1998). This viral protein targets IRF-7 for proteasomal degradation (Yu *et al.*, 2005), and more recently has been shown to mediate degradation of TRIF also by targeting it for proteasomal degradation (Ahmad *et al.*, 2011). The ORF K8 (K-bZIP) has been shown to inhibit IRF-3, but instead of direct interaction with the transcription factor, this viral protein binds efficiently to the PRDIII-I region of the IFN-β promoter, thus inhibiting the binding of the IRF-3-CBP/p300 complex (Lefort *et al.*, 2007). This strategy leads to inhibition of formation of a functional enhanceosome complex, thereby resulting in a defective transcription of IFN-β.

There are several IRF member homologues in KSHV. For example, the protein encoded by ORF K9 was described as the first viral member of the family, vIRF-1 (Moore *et al.*, 1996). The vIRF-1 protein interferes with the transactivation ability of both IRF-1 and IRF-3 by targeting a

common transcriptional cofactor; CBP/p300 (Burysek *et al.*, 1999; Lin *et al.*, 2001). The vIRF2 is also an inhibitor of the expression of IFN inducible genes, which are regulated by IRF-1, IRF-3 and ISGF3, but not by IRF-7 (Fuld *et al.*, 2006). In addition, this viral protein also interacts with PKR and thereby prevents the antiviral effects mediated by PKR (Burysek & Pitha, 2001).

The KSHV has also evolved multiple strategies to avoid host immune responses during latency, including antagonizing type I IFN signaling pathways. Examples of these are the expression of latent proteins such as LANA, which inhibits function of IRF-3 (Cloutier & Flamand, 2010) and vIRF-3, which binds to IRF-7 (Joo *et al.*, 2007).

In the case of HCMV infection, the mechanisms for subversion of interferon have been extensively studied. However, only a small number of genes have been identified as being responsible for modulation of the interferon response. The immediate-early proteins of HCMV are the obvious candidates, as they are the first genes being expressed, and indeed, the IE72 protein has been shown to play a role in inhibiting the antiviral state by binding to promyelocytic leukemia (PML) protein and disrupting PML-associated nuclear bodies (NBs) leading to the displacement of PML-NB associated proteins such as PML, Sp100 and Daxx (Ahn *et al.*, 1998; Ahn & Hayward, 1997; Korioth *et al.*, 1996; Wilkinson *et al.*, 1998). In addition, IE72 also binds to Stat2, and to a lesser extent, to STAT1, thereby inhibiting the IFN signaling pathway (Huh *et al.*, 2008; Paulus *et al.*, 2006). The IE86 protein has been described as an inhibitor of IFN-β production by blocking NF-κB (Taylor & Bresnahan, 2005). On the other hand, UL83 has been also shown to inhibit IFN-β production, but in this case by inhibiting IRF-3 phosphorylation and translocation into the nucleus (Abate *et al.*, 2004). HCMV also encode two PKR antagonists, proteins IRS1 and TRS1 (Cassady, 2005; Hakki & Geballe, 2005). Recently, an HCMV protein without known function, ORF94, was identified as an inhibitor of OAS expression during infection, therefore limiting the induction of OAS-mediated antiviral response (Tan *et al.*, 2011).

In order to replicate and persist, the HSV-1 virus, like the above described herpesviruses, also elicits innate immune responses just after virus entry - for example, through activation of IRF-3 (Preston *et al.*, 2001). Such evasion strategies include the expression of genes that target the IRF-3 signaling pathway, thereby inhibiting the production of IFN type I, and interfering with the signaling through the receptors and inhibiting the anti-viral state. Examples of such early expressed HSV-1 evasion genes include ICP0 and ICP27 which already had a described function, but were subsequently found to also inhibit IRF-3 accumulation in the nucleus (Eidson *et al.*, 2002; Melroe *et al.*, 2004), and to inhibit STAT1 phosphorylation and its subsequent translocation to the nucleus (Johnson & Knipe, 2010; Johnson *et al.*, 2008), respectively. Another HSV-1 protein, ICP34.5, was identified as playing a role in the PKR signaling pathway due to its sequence homology with mouse MyD116 (He *et al.*, 1998; He *et al.*, 1997). More recently, ICP34.5 has been found to form a complex with TBK1, thereby disrupting the interaction between TBK1 and IRF-3 (Verpooten *et al.*, 2009). Finally, US11 and UL41 have been shown to: bind to PKR (Cassady *et al.*, 1998), to block the activation of OAS (Sanchez & Mohr, 2007); and to mediate the degradation of cellular proteins such as ISGs (Kwong & Frenkel, 1989; Matis & Kudelova, 2001; Paladino & Mossman, 2009).

2.5 The herpesviruses viral protein kinases family

All of the α-, β- and γ-herpesviruses genomes contain several genes that are conserved between families. One of these is the protein kinase family, which includes ORF36 from KSHV, UL13 from HSV, BGLF4 from Epstein-Barr virus, and UL97 from HCMV.

Conservation of these viral protein kinase domains among the herpesviruses suggests that they are indispensable for survival of herpesviruses. The HCMV UL97 protein was the first to be defined and described as a protein that phosphorylates the antiviral nucleoside analogue ganciclovir, and therefore, could be a useful tool in the understanding of the antiviral activity of new selective anti-HCMV compounds (Littler *et al.*, 1992). Subsequently, other groups demonstrated that UL97 is an auto-phosphorylating serine-threonine kinase (He *et al.*, 1997). Along with a conserved role, these individual kinases may have unique functions in the context of viral infection. For example, the inhibition of the activity of HCMV UL97 protein kinase by a number of compounds that exhibit a pronounced antiviral effect is not shared by other protein kinases, such as BGLF4, illustrating the fact that low homology between the members of this group complicates the design of compounds able to target all herpesviruses, and suggesting that structure-based inhibitor designed for each group of herpesviruses might be more effective. On the other hand, Cyclopropavir (CPV), with a mechanism of action similar to ganciclovir, is active against HCMV as well as both 28 variants of HHV 6 and KSHV (James *et al.*, 2011).

The kinase domain containing KSHV ORF36 homologue was tested, and indeed, not only displayed an intrinsic protein kinase activity but was also autophosphorylated on a serine residue (Park *et al.*, 2000). Several studies have demonstrated that these conserved herpesvirus protein kinases impact at multiple steps in the virus life cycle, such as regulation of viral and cellular genes, nuclear egress, virus maturation and replication, chromosome condensation, and tissue tropism (Asai *et al.*, 2007; Gershburg & Pagano, 2008; Hamza *et al.*, 2004; Izumiya *et al.*, 2007; Kawaguchi & Kato, 2003; Michel & Mertens, 2004).

The ORF36 proteins from KSHV, HSV (UL13), HCMV (UL97) and Epstein-Barr virus (BGLF4) localize predominantly in the nucleus of cells (Daikoku *et al.*, 1997; Marschall *et al.*, 2005; Park *et al.*, 2000), and are only expressed several hours post-infection, with an early-late kinetics (Martinez-Guzman et al, 2003). However, due to the fact that they are present in the virion (Overton *et al.*, 1992; Varnum *et al.*, 2004; Zhu *et al.*, 2005), they are released into the cytoplasm of host cells just after virus entry and play essential roles as innate host evasion proteins. Until recently, this conserved herpesviruses family of protein kinases did not have any assigned function. Further experiments, however, showed that the UL97 viral protein kinase was able to mimic cdc2 in infected cells by targeting the same phosphorylation site in eukaryotic elongation factor 1 delta (Kawaguchi *et al.*, 2003). In addition, ORF36 from KSHV was showed to be involved in the activation of c-jun N-terminal kinase (JNK) pathway (Hamza *et al.*, 2004). The ORF36 from MHV-68, a murine gamma-herpesvirus, was also found to phosphorylate histone H2AX during infection, suggesting that the virus actively initiates and benefits from the host DNA damage response (Tarakanova *et al.*, 2007b), a function that is only shared by BGLF4 and not by the other homologues. Recently, phosphorylation of H2AX has been shown to correlate with gamma-herpesviruses latency *in vivo* (Tarakanova *et al.*, 2010a).

More recently, however, ORF36 from the murine gamma-herpesvirus, MHV-68, has also been shown to suppress the host IFN mediated response by inhibiting the transcription factor IRF-3 (Hwang *et al.*, 2009). This function, although not requiring the conserved kinase domain, is shared by the other homologues. The ORF36 protein binds directly to IRF-3, in a region that is required for CBP binding to IRF-3. In the signaling pathway that leads to the activation of IFN-β, both CBP and Pol II are recruited to the promoter region, and when ORF36 is expressed, this recruitment, mediated by activated IRF-3, is impaired (Hwang *et*

al., 2009). The BGLF4 kinase was also shown to suppress the IRF-3 signaling pathway by inhibiting binding of IRF-3 to the IFN-β promoter (Wang *et al.*, 2009). In addition, studies by others (Jong *et al.*, 2010) have identified RNA helicase A (RHA) as an interacting partner of KSHV ORF36.

The principle focus of our work is on KSHV, which, as described above, has evolved many genes for the inhibition of type I IFN responses. Multiplicity of these genes reflects the importance of inhibiting IFN responses for virus survival. Specifically, we have screened the non-homologous genes for an inhibitory impact on the IFN response using reporter assays detecting activation of the critical transcription factors in particular conserved non-homologous herpesvirus genes, as they conservation suggests that their function must be important for virus survival and spreading. The ORF36 is indeed one such gene and, as already described, interacts directly with IRF-3 and inhibits IFN-β expression. When screened in ISRE and GAS reporter assays ORF36 inhibited both type I and type II IFN signaling cascades (Our unpublished results). The only other KSHV gene described so far to be interfering with the Jak-Stat signaling pathway is the IL-6 viral homologue, which play a role in the IL-6/STAT3 pathway. Our work raises the possibility that signaling through the type I and type II IFN receptors is inhibited by the ORF36 protein through a mechanism involving RHA and CBP. This is due to our observations that suggest that ORF36 does not impact directly on the STAT1 and STAT2 signaling transcription factors, but instead seems to be targeting a molecule downstream in the signaling cascade, perhaps one involved in the regulation/formation of the transcription complex at the promoter regions of both ISRE and GAS sequences, such as CBP or RHA.

We have also demonstrated that ORF36 of KSHV inhibited the expression of genes controlled through ISRE and GAS sequences in their promoter regions upon stimulation with IFN-α and IFN-γ, respectively. This inhibition was dependent on the kinase domain of ORF36, as its mutation reverted its inhibitory activity in both type I and type II IFN responses. As the STAT transcription factors are key regulators in this signaling pathway, we decided to investigate if the expression of ORF36 was interfering with the activation and subsequent nuclear translocation of STAT 1 and STAT2 after treatment of cells with IFN-α and IFN-γ. Both STATs were expressed at equal levels, and phosphorylated at specific residues in cells expressing ORF36 when compared to empty transfected cells after IFN treatment. However, the expression of the ORF36 protein did not alter the localization of the STATs proteins before or after treatment of the cells with type I and type II IFN. Thus both STAT1 and STAT2 similarly translocated to the nucleus upon IFN treatment in cells with and without expression of ORF36. These results suggest that the ORF36 protein may play a role in the nucleus, although this will have to be confirmed.

In conclusion, ORF36 is yet another multifunctional virus host evasion molecule which has impact at different stages of the virus replication cycle. Finally, a comprehensive understanding of herpesvirus mediated manipulation of interferon is far from complete, but will certainly provide a better understanding of the virus-host interactions, and a possible route to novel control strategies.

3. The cell cycle and DNA damage

3.1 Cell cycle progression

The eukaryotic cell cycle is operationally divided into four phases: G1, S, G2, and M. Quiescent cells, which are metabolically active but not dividing, are in the G0 phase, outside

the cell cycle. The three DNA damage-induced cell cycle checkpoints, G1/S, intra-S, and G2/M, are critical points in the cell cycle that monitor the integrity of the genome, leading to repair or programmed cell death if DNA damage is detected. The DNA damage sensor molecules that activate the checkpoints and the signal transducer molecules, such as protein kinases and phosphatases, appear to be shared by the different checkpoints to varying degrees. The specificity of the checkpoint is due to the effector proteins, which inhibit phase transition (Sancar *et al.*, 2004).

The G1/S checkpoint prevents cells from entering the S phase in the presence of DNA damage by inhibiting the initiation of replication. In the case of double strand breaks, the kinase ATM initiates the checkpoint pathway by phosphorylation of Chk2, which, in turn, will phosphorylate Cdc25A, thereby causing its inactivation and consequent accumulation of the phosphorylated (inactive) form of Cdk2. If the DNA damage is caused by UV light, phosphorylation of Cdc25A is mediated by ATR-Chk1 proteins. In either case, this rapid response is followed by the p53-mediated maintenance of G1/S arrest. During this phase there is an ATM-mediated increase in the level of p53 due to an increase in protein stability, followed by posttranslational modifications, such as phosphorylation at amino acid residues 15 and 20, which regulate p53 functions. A key target for transcriptional activation of p53 is the cyclin-dependent kinase inhibitor, p21. The p53-dependent increase in p21 expression inhibits cyclin E and cyclin A-associated Cdk2 activities preventing G1-to-S phase progression (Lukas *et al.*, 2004; Sancar *et al.*, 2004).

The intra-S phase checkpoint is activated by damage encountered during the S phase or by unrepaired damage that escapes the G1/S checkpoint and leads to a block in replication. When double strand breaks are detected in S phase, the ATM-dependent pathway is activated leading to proteosome-mediated degradation of Cdc25A and consequently to the failure to maintain activation of cyclin-Cdk2 complexes and thus inhibition of DNA synthesis. A block of replication fork progression by intrinsic events or environmental insults, on the other hand, triggers an ATR-dependent pathway. In this latter case, members of the Rad family of checkpoint proteins function as DNA damage sensors and as scaffolds for the assembly of signaling complexes (Abraham, 2001).

The G2/M checkpoint prevents cells from undergoing mitosis in the presence of DNA damage, which could lead to the propagation of unrepaired DNA to the next generation. Double mutations of ATR and ATM completely eliminate G2 arrest following DNA damage, suggesting that both kinases are key regulators of this checkpoint. Thus, depending on the type of DNA damage, the ATM-Chk2 and/or ATR-Chk1 pathway is activated to arrest the cell cycle in G2 phase (Sancar *et al.*, 2004). Both pathways converge on the Cdc25C phosphatase. The Cdc2 catalityc subunit is inhibited by its phosphorylation at Thr14/Tyr15. Dephosphorylation of Cdc2 inhibitory sites by Cdc25C activates Cdc2-Cyclin B1 complex, allowing progression of cell cycle to mitosis. The activity of Cdc2 is also regulated by the availability of the cyclin B subunit. During S phase, cyclin B1 mRNA and protein begin to accumulate and their levels are maximal at G2/M. As cells pass through mitosis, cyclin B1 is ubiquitinated and degraded (Lou & Chen, 2005; Sancar *et al.*, 2004).

3.2 Herpesvirus modulation of the cell cycle

Replication of DNA viruses in host cells triggers a variety of cellular signaling cascades that regulate cell cycle, including the DNA damage response. It is generally accepted that viruses modulate the cell cycle to promote a transition through G1-S phase and achieve the cellular

environment for productive virus replication (Sato & Tsurumi, 2010). The herpesviruses genome, in contrast to other small DNA viruses, encodes a viral DNA polymerase and accessory factors, so it is not essential to promote entry into S phase of the cell cycle to exploit cellular genes involved in DNA replication. The interaction between herpesviruses and cell cycle regulatory mechanisms is more complex as some viral factors elicit cell cycle arrest while others promote cell cycle progression. Thus, it seems that herpesviruses induce cell cycle arrest, but they are able to block it in the cell cycle phase which most favors viral replication, perhaps to avoid competition with cellular DNA replication (Flemington, 2001).
Examples of cell cycle regulation by herpesviruses can be found in all three α-, β-, and γ-*herpesvirinae* subfamilies.
Herpes simplex virus type 1 (HSV-1) infection disrupts cell cycle progression in two different ways depending on the cell cycle phase. In quiescent cells, HSV-1 infection prevents G1 entry by inhibition of cyclin D/CDK4,6-specific and cyclin E/CDK2-specific phosphorylation of the retinoblastoma protein pRb. On the other hand, HSV-1 induces G1/S arrest in dividing cell cultures by inhibition of preexisting cyclin E/CDK2 and cyclin A/CDK2 activities (Ehmann *et al.*, 2000; Song *et al.*, 2000). The ability of HSV-1 to alter cellular environment in order to enhance viral replication has been related to the expression of immediate-early protein ICP0. Infection with a mutant virus in which ICP0 is the only IE protein expressed resulted in p53-independent cell cycle arrest in G1/S and G2/M phase. Although ICP27 also plays a key role in the activation of G1/S phase (Song *et al.*, 2001), infection with different mutant viruses demonstrated that the mitotic block requires ICP0 expression (Hobbs & DeLuca, 1999; Lomonte & Everett, 1999).
Similar to HSV-1, several studies demonstrated that HCMV infection leads to drastic and temporally coordinated alterations in the expression of host cell regulatory proteins, such as cyclins, resulting in cell cycle arrest at more than one phase (Bresnahan *et al.*, 1996; Dittmer & Mocarski, 1997; Jault *et al.*, 1995; Lu & Shenk, 1996). The cell cycle arrest was independent of the cell cycle phase (G0, G1, and S) at the moment of infection with HCMV (Salvant *et al.*, 1998). The immediate-early proteins IE1/IE2 and UL69 have been described as being able to block cell cycle in G1, suggesting that they may have a role in HCMV manipulation of cell cycle progression (Castillo *et al.*, 2005; Lu & Shenk, 1996; Wiebusch & Hagemeier, 1999). Infection with UV-irradiated HCMV virus resulted in G1 arrest when cells exit from G0, but it was unable to block the S phase entry in cycling cells, indicating that gene expression is required and excluding viral capsid proteins, such as UL69, function. Thus, it seems that under different conditions, these proteins may have a higher or lower impact on cell cycle regulation by HCMV (Wiebusch & Hagemeier, 1999).
The γ-herpesvirus EBV lytic program promotes its own specific cell cycle-associated activities involved in the progression from G1 to S phase, while at the same time inhibiting cellular DNA synthesis. Although the levels of p53 and CDK inhibitors remain unchanged throughout the lytic infection, there is an increase in the amounts of cyclin E/A and the hyperphosphorylated form of pRb. The resultant "quasi" S-phase cellular environment is essential for the transcription of viral immediate-early and early genes, probably due to the availability of transcription factors, such as E2F-1 and Sp1, which are expressed during S phase (Kudoh *et al.*, 2004; Kudoh *et al.*, 2003). The EBV gene which has a relevant role in cell cycle modulation is the immediate early transcription factor Zta, also known as ZEBRA or BZLF1. The Zta protein mediates the induction of three key cell cycle regulatory factors, p21, p27, and p53, resulting in G0/G1 cell cycle arrest in several epithelial cell lines (Cayrol &

Flemington, 1996). Interestingly, Zta mediates the induction of each one of these proteins, in part, through distinct pathways, a strategy which may have evolved to ensure that lytic replication occurs in a growth-arrested setting in different tissues in various states of differentiation (Rodriguez *et al.*, 1999). This emphasizes a fundamental aspect of pathogen-host evasion, the logical tailoring of the host evasion mechanism to the life style of the pathogen.

Recent studies with HHV-6A, one of the less studied β-herpesviruses, demonstrated that infection of T cells with HHV-6A results in cell cycle arrest at the G2/M phase. In this case, G2 arrest may serve to block the clonal expansion and proliferation of HHV-6A specific T cells to maintain immune suppression and evade the antiviral immune response (Li *et al.*, 2011). The mechanism that regulates the block in cell cycle progression by HHV-6A involves the inactivation of the cdc2-cyclin B1 complex and the increased expression of p21 protein in a p53-dependent manner. Manipulation of the cell cycle was also described for HHV-6B (Øster *et al.*, 2005) and HHV-6A infection in cord blood mononuclear cells (De Bolle *et al.*, 2004), but with different results, suggesting that the regulatory pathways and mechanisms induced by HHV-6 infection might be different according to the type of infected cells, once again correlating host evasion with host biology.

The regulation of cell cycle by herpesviruses is closely related to activation of the DNA damage response, including double strand break repair pathways. Viral infection confronts the host cell with large amounts of exogenous genetic material that might be recognized as abnormal and damaged DNA and so precipitates the premature apoptosis of the virus infected cells (Weitzman *et al.*, 2004). During replication, herpesvirus DNA is synthesized in a rolling-circle manner to produce head-to-tail concatemers that are subsequently cleaved into unit-length genomes. These too may be recognized as double strand breaks and trigger a DNA damage response (McVoy & Adler, 1994). Thus, in order to establish a productive infection, it is essential that viruses defend themselves from the host cell DNA damage response machinery. Paradoxically, recent reports indicate that such cellular responses may have a beneficial role in viral replication (Luo *et al.*, 2011).

Thus, during HSV-1 infection, RPA, RAD51, NBS1, and Ku86 components of the HR pathway are recruited to replication compartments, suggesting that homologous repair may play a role at the earliest stages of HSV-1 DNA replication. The phosphorylation of NBS1 and RPA indicates, in addition, that HSV-1 infection may activate components of the DNA damage response. Although siRNA-mediated "knock down" studies indicated that ATM is not absolutely required for HSV DNA replication and infectious virus production (Shirata *et al.*, 2005), another report has demonstrated that absence of specific DNA repair proteins, such as ATM or MRN complex, result in significant defects in viral replication, suggesting that a DNA damage response environment is beneficial for productive HSV-1 infection (Lilley *et al.*, 2005). Consistent with the HSV-1 infection studies, the mechanism of cell cycle arrest by ICP0 requires ATM and Chk2 proteins, and results from the activation of ATM-Chk2-Cdc25C DNA damage pathway (Li *et al.*, 2008).

The DNA damage response is also activated during HCMV replication, as indicated by the phosphorylation of ATM and H2AX and the downstream proteins Chk2 and p53. However, during HCMV infection, the localization of various checkpoint proteins, normally organized near the site of damage, is altered, inhibiting their normal function. Importantly, recent results indicate that the DNA damage response mediated by E2F1 transcription factor contributes to replication of HCMV (E *et al.*, 2011).

The EBV BGLF4 kinase may be relevant for EBV-induced DNA damage, as it induces H2AX phosphorylation. This function, conserved in the MHV-68 homolog (ORF36), is dependent of the kinase domain and is enhanced by ATM (Tarakanova *et al.*, 2007a). Recently, it has been demonstrated that ORF36 and H2AX have an important role in the establishment of MHV-68 latency, suggesting that γ-herpesviruses may exploit the components of DNA damage responses, not only during lytic replication, but also during latent infection *in vivo* (Tarakanova *et al.*, 2010b).

It is still not clear if virus-induced DNA damage involves the recognition of existing double strand breaks. Thus, it is possible that the trigger of herpesvirus-induced DNA damage response is not the recognition of viral DNA as double-strand breaks, or actual damage to DNA, but is the recruitment of DNA damage repair factors observed during viral infection.

Understanding the interaction between the DNA damage response machinery and virus infections will not only provide insights into viral pathology and persistence, but also new ideas for the development of antiviral and anti-tumour drugs. Moreover, knowledge of virus–host interactions can elucidate the mechanisms of key cellular control processes and help in identifying as yet unrecognized signaling pathways. Thus, the study of virus host evasion mechanisms can provide new tools to study recognition and repair of damaged DNA by cellular machinery.

3.3 The UL24 family: Multifunctional virus host evasion proteins

The UL24 gene is located in the unique long segment of HSV-1 genome, overlapping the thymidine kinase gene (McGeoch *et al.*, 1988). It is conserved not only in all three subfamilies of human herpesviruses, but also in other mammalian, avian, and reptilian herpesviruses, with exception of amphibians or fish, all of which have a distinct relationship to all subfamilies. Of the core herpesviruses genes, UL24 is the only one that remains unassigned to any functional category (Davison *et al.*, 2002); its universal presence in herpesviruses and lack of homology with cellular genes suggests that UL24 gene family has a relevant role in the viral life cycle and/or host evasion mechanisms. The UL24 homologues are generally expressed with late kinetics, although HSV-1 UL24 has a complex transcription pattern with leaky-late kinetics since its expression is not completely dependent on viral replication (Pearson & Coen, 2002). The HSV-2 UL24 and HCMV UL76 homologues, however, have unambiguously been identified as virion-associated proteins, and so they are found within the cell from the moment of infection (Hong-Yan *et al.*, 2001; Wang *et al.*, 2000). The alignment of the predicted amino acid sequences of UL24 homologues reveals five regions of strong sequence similarity (Jacobson *et al.*, 1989). Interestingly, a comparison of sequence profiles, enriched by predicted secondary structure, has identified UL24 as a novel PD-(D/E)XK endonuclease belonging to a large superfamily of restriction endonuclease-like fold proteins (Knizewski *et al.*, 2006). To date, however, no nuclease activity has been reported for UL24 or any of its homologues.

Deletion of the UL24 gene in HSV-1 resulted in a virus with significantly reduced plaque size and associated decreased viral yield, suggesting that the UL24 function, although not essential, is important for virus growth, at least in cell culture (Jacobson *et al.*, 1998; Jacobson *et al.*, 1989). Similar results were obtained for HSV-2 UL24 (Blakeney *et al.*, 2005), VZV ORF35 (Ito *et al.*, 2005), MHV-68 ORF20 (Nascimento & Parkhouse, 2007), and HCMV UL76 from AD169 strain (Yu *et al.*, 2003). Global functional analysis conducted using HCMV Towne complete genome, however, indicated that UL76 is an essential gene for viral

replication (Dunn *et al.*, 2003). Virus strains with substitution mutations in the endonuclease motif of UL24 exhibited titers in the mouse eye model that were 10-fold lower than the wild-type virus and similar results were obtained in trigeminal ganglia. Furthermore, the percentage of virus reactivation was also significantly lower. These results are consistent with the endonuclease motif being important for the role of UL24 *in vivo* (Leiva-Torres et al., 2010). Further deletion studies revealed that absence of UL24 from HSV-1, HSV-2 and VZV leads to a syncytial plaque phenotype, similar to that observed for other HSV-1 viral proteins, suggesting that UL24, like UL20 and gK, may have a role in viral egress (Blakeney *et al.*, 2005; Ito *et al.*, 2005; Pearson & Coen, 2002), although the possible involvement of UL24 in assembly and egress of virus particles remains to be explored.

During viral infection, UL24 homologues are detected predominantly in the nucleus, and transiently localize in the nucleoli (Hong-Yan *et al.*, 2001; Nascimento & Parkhouse, 2007; Pearson & Coen, 2002; Wang *et al.*, 2000). Indeed, infection with HSV-1 results in dramatic alterations to nuclear structure and organization, including the morphology of nucleoli (Besse & Puvion-Dutilleul, 1996). Moreover, in cells infected with HSV-1, in contrast to the large, prominent spots observed within the nucleus of mock-infected cells, nucleolin had a diffuse distribution throughout the nucleus. Significantly, cells infected with two independent UL24-deficient viruses, UL24XB and UL24XG, retained the prominent punctate foci of nucleolin revealed by staining, although not absolutely identical to the distribution seen in uninfected cells.

Thus UL24 must contribute to the observed alteration of the nucleoli in HSV-1 infected cells (Lymberopoulos & Pearson, 2007), a conclusion reinforced by experiments demonstrating that expression of the N-terminal domain of UL24 alone is sufficient to induce the redistribution of nucleolin in the nucleus (Bertrand & Pearson, 2008). A similar impact of UL24 on nucleolar morphology was observed with B23, another abundant nucleolar protein, but not with fibrillarin, which also assumes a diffuse nuclear distribution as a consequence of HSV-1 infection, but in a UL24-independent manner (Callé *et al.*, 2008; Lymberopoulos *et al.*, 2011). Since UL24 is implicated in the redistribution of B23 and nucleolin, it is possible that it plays a role in nuclear egress through its effect on nucleoli, although how nucleolin affects HSV-1 nuclear egress is obscure. Finally, the fact that deletion of UL24 conserved homology domains, including the putative endonuclease motifs, resulted in loss of nucleolin and B23 dispersal activity, suggests that this function may be shared among all herpesviruses and must be relevant for the viral life cycle (Bertrand *et al.*, 2010; Bertrand & Pearson, 2008; Lymberopoulos *et al.*, 2011).

The first results that demonstrated UL24 involvement with cell cycle manipulation were obtained for ORF20 protein, the UL24 homologue from MHV-68 (Nascimento & Parkhouse, 2007). This virus is a particularly useful model for the study of herpesvirus *in vivo*. Similar to the human homologues, ORF20 is located in the nucleus and, when transiently expressed in human and murine cell lines, it induces cell cycle arrest at the G2/M phase, followed by apoptosis at later time points. During the G2 phase, the cyclin B/Cdc2 complex is inactive as the Cdc2 protein is in the phosphorylated form. Consistent with the observed G2 arrest, cells expressing MHV-68 ORF20 showed an increased phosphoryation of Cdc2 at the inhibitory site Tyr15 and a consequent inactivation of Cdc2-cyclin B complex was demonstrated (Nascimento & Parkhouse, 2007). As ORF20 belongs to UL24 gene family, the possibility that other homologues might have a role in cell cycle modulation was explored. In fact, the UL24 homologues from human herpesviruses representative of each subfamily

(HSV-1 UL24, HCMV UL76 and KSHV ORF20) also induced cell cycle arrest followed by apoptosis. Moreover, and as observed for MHV-68 ORF20, the UL24 homologues expression also resulted in Cdc2 phosphorylation at the Tyr-15 inactivation site and consequent inhibition of the mitotic cdc2-cyclin B complex (Nascimento *et al.*, 2009).

The precise mechanism of cell cycle arrest induced by UL24 homologues remains to be clarified. An interesting and possible explanation is the recent report that HCMV UL76 induces chromosomal aberrations and DNA damage (Siew *et al.*, 2009). Phosphorylation of ATM and γ-H2AX, accepted signals of DNA damage activation, were also observed for HSV-1 UL24 (our unpublished work), possibly revealing a conserved effect similar to the observed cell cycle arrest. It may be relevant that the number of cells with DNA damage breaks increased with increasing levels of the UL76 protein, a feature perhaps related to the putative endonuclease activity of the viral protein (Siew *et al.*, 2009).

Another example of this multi-functionality of virus host evasion proteins is the induction of the expression of interleukin-8 (IL-8) by the HCMV UL76 gene (our unpublished work). Thus the up-regulation of IL-8 observed in infections with HCMV (Murayama *et al.*, 1997), HSV-1 (Li *et al.*, 2006), KSHV (Lane *et al.*, 2002), and EBV (Klein *et al.*, 1996) is consistent with the properties of the conserved herpesvirus host evasion gene, UL24 gene family. The pro-inflammatory chemokine IL-8 is a member of the CXC chemokine family that binds with high affinity to two chemokine receptors, CXCR1 and CXCR2 (Zlotnik & Yoshie, 2000) and is predominantly chemotactic for neutrophils. Following infection, HCMV spreads via the bloodstream to various organs. During this phase, infectious HCMV is detected in all major leukocyte populations, with neutrophils being the most frequent reservoirs of viral DNA.

Although the virus may not replicate within them, neutrophils play an important role in the dissemination of HCMV throughout the body. Significantly, IL-8 expression is up-regulated in HCMV infected cells (Craigen *et al.*, 1997), and its secretion has direct consequences as it increases the production of infectious virus (Murayama *et al.*, 1994). Thus the stimulation of IL-8 expression appears to be a virus strategy evolved to enhance virus dissemination and survival, playing a key role in the pathogenesis of the infection. In summary, it is interesting that activation of DNA damage by UL76, a single viral gene, without cellular homology, yet conserved in all herpesviruses, has two very different activities, cell cycle manipulation and IL-8 induction, both very important for HCMV life cycle and host evasion. Whether these two activities are the consequence of two entirely different functional domains, or are in some way related, remains to be seen.

To further understand the role of UL24 on viral pathogenesis *in vivo*, we constructed two independent ORF20 deletion mutant MHV-68 viruses. Since MHV-68 infects both outbred and inbred mice, MHV-68 is a useful model to study the lytic and latent phase of herpesvirus infection and consequent pathogenesis *in vivo*. After intranasal inoculation of mice, MHV-68 replicates in lung epithelial cells. This lytic phase of infection is resolved within 10-12 days in mice infected with wild-type virus. In contrast, in mice infected with the two independent mutants for ORF20, the virus titers in the lungs were still elevated after ten days post-infection, whereas it was almost undetectable, at the same time point in the mice infected with wild-type MHV-68 and revertant viruses. However, fifteen days after infection, the lytic phase in mice infected with ORF20 deficient viruses was resolved and the virus established latency in the spleen. The fundamental cause of the extended viraemia in mice infected with ORF20-mutant viruses remains to be elucidated. After the initial lytic virus replication in the lungs, MHV-68 establishes latency in B lymphocytes in the spleen. In

contrast to the extended acute phase of ORF20 mutant viruses, no major difference was found in the reactivation from latency of mutant virus compared to MHV-68 wild type infection (Nascimento *et al.*, 2011).

Other studies *in vivo,* using UL24 homologues from HSV-2 or VZV, also suggest a critical role for UL24 in viral pathogenesis. Intravaginal inoculation of BALB/c mice and Hartley guinea pigs with a UL24 deletion mutant of HSV-2 virus resulted in delayed disease kinetics and minimal disease progression, and the mutant virus was avirulent at higher doses compared to the parental virus (Blakeney *et al.*, 2005). Deletion mutant VZV virus infection of skin and T cells xenografts *in vivo* also indicated that ORF35 is a determinant of VZV virulence (Ito *et al.*, 2005). It would be important to analyze the ORF20 mutant MHV-68 pathogenesis in its natural host, the wood mice (members of the genus *Apodemus*) (Ehlers *et al.*, 2007), as a recent comparative analysis revealed that MHV-68 infection of BALB/c (*M. musculus*) and laboratory-bred wood mice are markedly different (Hughes *et al.*, 2010).

In conclusion, manipulation of the DNA damage response is a major target of herpesviruses, and one that in turn may determine a variety of significant outcomes to favor the virus, with cell cycle arrest and induction of IL-8 now identified.

4. Conclusion

Many virus genes that manipulate host defense mechanisms are recognized by their homology to cellular genes. Viruses, however, contain many genes without any homology or known function in the now vast sequences database. As all the structural proteins and most of the enzymes of herpesvirus are now known, it is likely that a considerable number of their non-assigned, non-homologous genes have evolved for host manipulation, thus offering opportunities for other novel strategies for control of virus infection, such as construction of a gene deletion, avirulent virus vaccines. Indeed, as we report here, the UL24 gene family, which is conserved in all herpesviruses, and has no cellular homologue, combines the dual function of inhibiting cell cycle progression and inducing IL-8 expression.

Significantly, both UL24 and the kinase ORF36 are genes conserved in α-, β- and γ-herpesviruses, and both share the function of activating the DNA damage pathway. At the same time, both UL24 and ORF36 impact on innate immunity, the first inducing expression of IL-8, and the second inhibiting IFN responses. Thus UL24, like many other virus host evasion genes, is multifunctional, impacting on two cellular activities, the cell cycle and IL-8 expression. Although at first site this particular combination of targets for one viral gene is not an obviously rational one, future studies may provide the logical mechanistic link for many more multifunctional virus host evasion genes.

In conclusion, non-homologous virus genes may be an Aladdin's cave of "ready-made tools" for the manipulation of cell biology and immune responses, and the multi-functionality of virus host evasion genes may provide important tools to unravel the complex interactions underlying the regulation of intracellular signaling.

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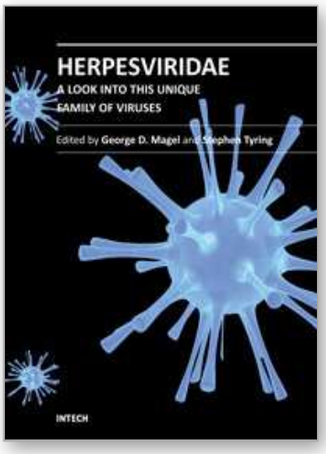

Herpesviridae - A Look Into This Unique Family of Viruses

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In order to fully understand the nature of viruses, it is important to look at them from both, their basic science and clinical, standpoints. Our goal with this book was to dissect Herpesviridae into its biological properties and clinical significance in order to provide a logical, as well as practical, approach to understanding and treating the various conditions caused by this unique family of viruses. In addition to their up-to-date and extensive text, each chapter is laced with a variety of diagrams, tables, charts, and images, aimed at helping us achieve our goal. We hope that this book will serve as a reference tool for clinicians of various specialties worldwide.

How to reference

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