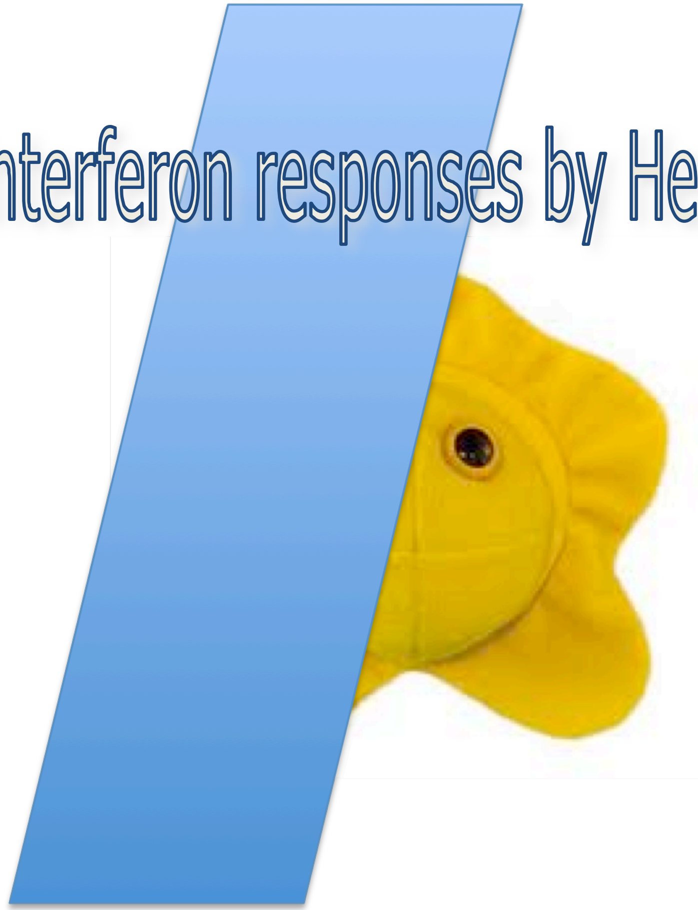
Evasion of interferon responses by Herpes viruses



Master Thesis

Infection & Immunity

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ABSTRACT

Viruses might be around as long as life itself. The core essence of these entities is their ability to tap in on the resources of their particular host, without being part of that host. From the very start of life (probably) this has put some evolutionary pressure on organisms; wherever they produce metabolites, energy, safe environments, etc. for parasitizing (viral) entities they are going to lose the evolutionary race from their peers who only produce for themselves. Organisms were therefore bound to develop defense mechanisms against viruses. The only true challenge for viruses is to circumvent these defenses.

Mammalian hosts have developed a highly effective anti-viral strategy that uses interferons (signaling molecules). The recognition of a virus by the wide arsenal of detection receptors that lead to the induction of interferons, invoke multiple effector and warning cascades. These cascades give rise to an anti-viral state of the cell and educate the professional (adaptive) killer cells.

Herpes viruses form an ancient class of human pathogens that have evolved a counter-arsenal to deal with the host defense strategies. This class of viruses is quite effective in their strategies to evade the host defenses, as they persist in the host.

This thesis provides part of the answer to the question how herpes viruses stay under the radar from the immune system. It focuses on the initial infection and the intracellular race between the interferon system and herpes virus evasion.

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PROLOGUE INTRODUCTION AND RESEARCH QUESTION

Upon viral infections, an innate and adaptive immune response is triggered. The specialized neutralization of viruses by antibodies generated in B-cells and the killing of virus-infected cells by CD8+ T-cells and NKT-cells are effective adaptive systems to clear viruses. One of the downsides of these tailored systems is their relatively long maturation period. Before a sufficiently high antibody titer directed against a particular virus is reached, the infection could be lethal.

Innate receptors and interferon type I

More than two decades ago, Janeway *et al*. appreciated the intrinsic ability of the mammalian immune system to recognize pathogen associated molecular patterns (PAMPs) by cellular receptors that were dubbed pathogen recognition receptors (PRRs). This discovery linked the innate immune system to the specific recognition of pathogens for the first time. A wide range of PPRs has been discovered since and new ones are still being reported. Several intracellular PPRs survey various compartments of the cell to start a signaling cascade when suspicious compounds are encountered. These cascades activate transcription factors inducing genes that function in a semi-tailored innate immune response.

Generally, the interferon (IFN) type I (IFN-α and -β) genes are main targets of PPR-induced signaling upon recognition of viral PAMPs. These signaling proteins in turn stimulate the expression of over three hundred different genes and therefore have a broad range of effects. Direct anti-viral proteins are upregulated, protein synthesis is diminished and chemokines are released. Also, IFN type 1 stimulates the maturation and antigen-presenting capacity of dendritic cells (DCs), by increasing the expression of CD86, CD80 and CD40. Additionally, it mediates the CD8+ specific T cell response (Kawai en Akira, Nat Immunol 2006). So before an adaptive immune response is invoked, a potent immune response is elicited in all cells that encounter a virus.

Cooperation between innate and adaptive immunity

The urge of men to categorize everything is also applied to the modes of action that the immune system has to fight of pathogens. In literature a clear distinction is made between innate and adaptive immunity on good and logic grounds. However, a total lack of synergy would be penalized by evolution. Indeed, cross-communication occurs between the PRR-mediated innate immune activation and the tailored adaptive immune response mediated by B-cells, CD8+ T-cells

and NK-cells. The priming of T-cells is an important step in their activation (licensing) and is often mediated by DCs. Uptake of antigens by DCs in order to present them to effector T-cells, as well as DC-activating signals from the surrounding tissue premise their ability to activate T-cells. DCs are able to load phagocytosed antigen in phagosomes and endosomes via a TAP-dependent and -independent mechanism. This so-called cross-presentation enables DCs to detect infections in any cell type (Le Bon 2008). The appreciation that type I IFN can activate DCs fulfills the second requirement for CD8+ T-cell priming (Tabi, 2001). This example provides a robust argument for the link between the innate and adaptive immune responses to intracellular pathogens. Additionally, type I IFN has stimulating and accelerating effects on the humoral response against several viruses (Coro 2006, Fink 2006).

Aim of this Thesis

Herpesviruses and human hosts have co-evolved, giving and taking battles, for millions of years. Each time that an emerged immune strategy promised to clear these viruses effectively, herpesviruses developed an evasive or counteractive mechanism to circumvent the immune strategy. This ongoing war between host and virus has produced an extensive set of genes – and strategies to apply these genes. One of them involves the type I IFN gene induction in the host, and suppressive countermeasures in the viruses. This thesis describes the interplay between host and virus concerning this issue.

PART I HUMAN HERPESVIRUSES

Herpesvirus pathogenesis

Herpesviruses are important human pathogens. Their symptoms range from skin blisters to fevers, enduring fatigue, and in very rare occasions lethal encephalitis. Some herpesviruses are the causative agents of several types of lymphomas or others tumors in immunocompromised patients. Their generally mild symptoms are stately compensated by their persistence and abundance, making herpesviruses an important issue for human health.

Lythic and latent expression of herpesviruses

One of the most characteristic features of herpesviruses in their infectious cycle, is the phase in which the virus is latently 'creeping' (literal translation of herpes from Greek) in the host. All (human) herpesviruses have the ability to retain in the host, even after the full immunological arsenal is put to practice. The possibility to run two types of expression programs is the main causative phenomenon for their persistence. All or most genes are expressed in the lythic program where active processes such as replication, immunomodulation, host shut-off, and assembly take place. Most other viruses express their genes following only this program. On the other hand, the latent expression program is unique to herpesviruses. During this phase of infection, the virus shuts off most of its own genes and camouflages its proteins and nucleic acids, while only expressing the bare minimum of genes to retain their genome. Usually, the cells that are infected by virus in the latent phase have undergone virus-induced immortalizing or are long lasting by nature. At any given time, in a small percentage of the latently infected cells the viruses change their expression profile to the lythic expression. When this lythic expression is not sufficiently controlled by the host, viral outbursts into symptom-accompanied disease can occur. Therefore, immunocompromised hosts can have trouble coping with these viruses, especially with the oncogenic γ-herpesviruses.

Taxonomy of herpesviruses

In the order of the Herpesvirales there are several families of which three contain a total of eight well-defined human pathogens; (α-)herpesviruses, β-herpesviruses and γ-herpesviruses. The herpesviruses contain the herpes simplex viruses-1 (HSV-1), HSV- 2, and the varizella zoster virus (also known as human herpesvirus (HHV)-3). The β-herpesvirus family also contains three known human pathogens, of which the human cytomegalovirus has the highest prevalence and is closely related to the murine cytomegalovirus. The murine cytomegalovirus (HVV-5) is an often-used model to investigate β-herpesviruses in laboratory settings. HHV-6 and HHV-7 are less well-studied members of the β-herpesviruses. Lastly, the γ-herpesvirus family comprises the

Epstein-Barr (HHV-4) virus (EBV) that is closely linked to the Rhesus lymphocryptovirus and the human Kaposi's sarcoma associated (HHV-8) virus (KSV). Most other known herpesviruses infect mammals, but some more distantly related herpesviruses infect birds and reptiles (reviewed in Davison, 2009).

Epidemiology of herpesviruses

The largest part of the world population is carrier of at least one herpesvirus. Over 90% is carrier of EBV, 35% carries HSV-1 and 40% to 90% is HCMV positive depending on the country(Rölle and Olweus 2009).

Structure and lifecycle of herpesviruses

Herpesviruses are double-stranded DNA viruses, which is linear in the virion. The DNA is packaged into nucleocapsid proteins that form an icosaheral structure in the centre of the virion. The core nucleocapsid is separated from the outer membrane layer by a proteineus tegument, which comprise several viral and host proteins, but also viral mRNAs. Several transmembrane (glyco)proteins span the outer membrane. Entry of HSV-1 is initiated by binding of glycoprotein B (gB) and gC to the extracellular matrix and subsequent binding of gD to a second receptor followed by membrane fusion. This releases the nucleocapsid and the tegument proteins and RNA to the cytosol. The localization to and release into the nucleus of the genome-containing nucleocapsid initiates transcription. In the herpesvirus lifecycle, three subsequent types of genes can be distinguished temporally during the lythic phase; immediate early (IE) genes comprise the tegument proteins and directly transcribed genes, early (L) genes follow the IE genes and late (L) genes are expressed thereafter. All three sets of genes have a specific function in the herpesvirus lifecycle. The IE proteins are primary focused on the quick onset of viral genome transcription and early immune evasion, whereas the late genes are more concerned with the generation and trafficking of new virus particles. The relatively large herpesvirus genomes, ranging from 125 to 290 kilobases (Davison 2009), encode approximately forty herpes proteins and at least twenty unique and genus specific genes. This ensures shared mechanisms within the genus, but also leaves much room of variation between specific herpesvirus processes.

Course of infection

HSV-1 and HSV-2 lytic outburst results from a retrograde trafficking of the virus to epithelial cells, from neurons in the spine where it retains in latency. In a mostly similar fashion Varicella Zoster causes an outburst later in life causing shingles, after a primary infection resulting in chickenpox. HCMV, EBV, and KSAV retain in lymphocytes (mainly B-cells) during latency. Primary infections can lead to fatigue due to the stressing of the immune system. Thereafter, the recurrent outburst into the lythic phase of a small percentage of the infected cells ensures their

transmissibility via oral or sexual contact. In general, in immuno-competent individuals the herpes viruses start off with a lythic infection, expressing the bigger part of their genes. Thereafter they persist in latency. Generally, when the virus breaks though latency it depends on cell-to-cell spread in order to infect new host cells. These new cells see the virus and can act on this, conceivably pre-informed by neighboring cells by means of IFN type I. This thesis focuses on primary infections and the lytic outbursts where most of the immune-evasive genes are expressed.

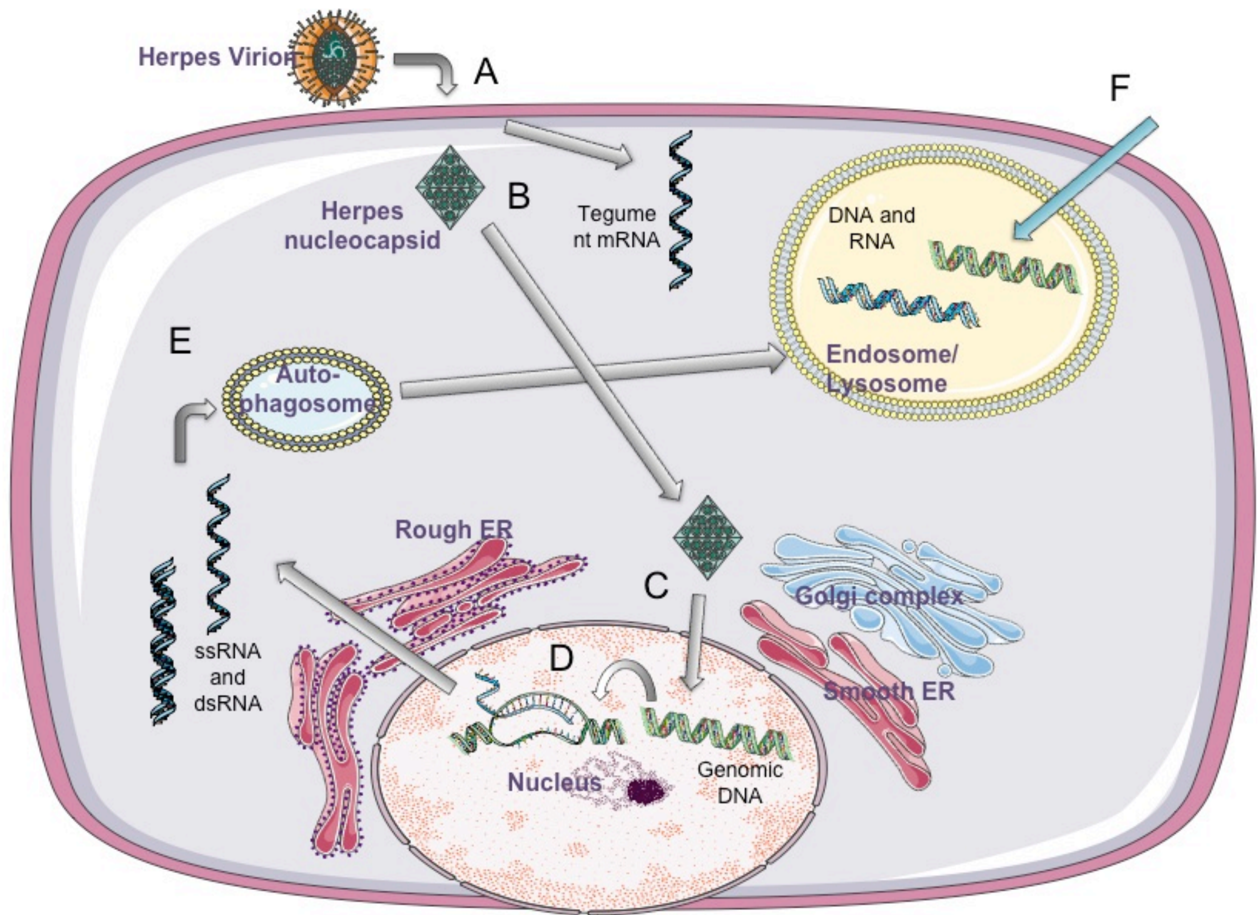

Figure 1. The lifecycle of Herpes Simplex Virus 1 (HSV1) reveals putative recognition events. (A) viral engagement of the cellular receptor can stimulate PRRs. (B) after fusion of the envelope with the plasma membrane, the nucleocapsid and several (immediate early) proteins and transcripts are released into the cytoplasm. These xenobiotic entities are potential stimulators of intracellular PRRs. (C) docking of the nucleoplasmid induces the release of the genome into the nucleus of the host cell, where transcription takes place (D). The released transcripts can be translated or recognized by PRRs. A normal degradative process is the uptake of transcripts and protein into autophagosomes (E), and transport/ maturation (in)to lysosomes for degradation. The PRRs in endosomes/ lysosomes sense the interior of these vesicles. (F) The nucleic acids that are taken up from the surroundings of the cell also end up in endosomes/ lysosomes.

PART II VIRUS RECOGNITION BY INTRACELLULAR INNATE MECHANISMS

Recognition systems

The functional implication of the previous is that there are many proteins that recognize viral motifs and thereby initiate signaling cascades that eventually lead to an antiviral state of the cell. During an infection, viral nucleic acids are of major importance in intracellular pattern recognition, as they can be profoundly different from host RNA and DNA in both composition (i.e. dsRNA) as well as localization (i.e. DNA in the cytosol). Two major classes of PRRs that can recognize viral nucleic acids can be distinguished; (i) the transmembrane Toll-like receptors (TLRs) that span the cytoplasmic membrane or the membrane of endosomes and (ii) RIG-I like receptors (RLRs) that are present in the cytosol. Both receptor families elicit IFN type I transcription.

Toll like receptors

PAMPs in endosomes

The previous appreciates the important role of foreign nucleic acid in virus detection. Viral nucleic acid can end up in the lumen of endosomes by way of three distinct events. The first and most important way is by uptake from the direct environment of the cell. This happens after virus-induced necrosis of neighboring cells, which then release their nucleic acid-containing interior. The second possibility depends on the viral entry mechanism. Many viruses enter the cell via induced endocytosis of which the downstream cellular components are endosomes and lysosomes. Some viruses are lysed before entering the cytosol, so the vesicular lumen is spilled into the endosomal lumen where TLRs recognize it. The third possibility occurs later in infection after the start of transcription of the viral genome. This uptake works through retrograde trafficking and can localize the nucleic acids in lumen of so-called autophagosomes (Lund, et al. 2003) (Kudchodkar and Levine 2009). Autophagosomes normally form around defective organelles or protein aggregates in the cytoplasm to remove them. These vesicles then fuse with endosomes and lysosomes and similarly viral components can be taken up and trafficked. Several TLRs can recognize nucleic acids in the lumen of endosomes.

TLR intracellular location

In humans, the TLR family of proteins comprises 10 members. These TLRs together can recognize various categories of PAMPs such as viral and bacterial proteins and motifs on the exterior of pathogens. They share extracellular or luminal leucine rich repeats which form the PAMP-recognizing domain. Consequently, TLRs cannot recognize pathogen in the cytoplasm or

the nucleus of infected cells. The cellular localization of the TLRs determines what type of PAMP it can encounter and hence recognize. Specifically, cell-surface TLRs have a different specificity than TLRs that have their leucine rich repeats (LRRs) in the lumen of intracellular components; TLR3, TLR7, TLR8 and TLR9. This gives the members of the two TLR groups their distinct niche in the PAMP-recognition machinery. TLR3, TLR7, TLR8 and TLR9 are co-translationally incorporated into the endoplasmic reticulum (ER) membranes, where they interact with the twelve-membrane-spanning protein UNC-93B. This trafficking protein delivers the TLRs for the ER to endosomes, thus it is required for the proper localization of this subfamily of TLRs. Normal functioning of UNC-93B in a prerequisite for proper TLR functioning, as mice that are deficient in Unc-93b show an impaired immune response to TLR3 and TLR9 ligands (Tabeta, et al. 2006).

Besides the luminal TLRs that are usually activated by viral infection, at least two plasma membrane TLRs have the capability to induce a response to viruses. TLR2 surveys the environment of the cell and in activated upon recognition of its ligand, which are in many cases whole virions or glycoproteins (Ariza, et al. 2009). TLR2 plays a role in the recognition of HCMV probably due to binding of gB and gH viral proteins (Marshall and Geballe 2009). There is also evidence for the involvement of TLR4 in the response to the envelope of some viruses (Boo, 2010).

Specificity

The four endosomal TLRs have been found to mainly recognize nucleic acids. The dogma about these receptor is that TLR3 is primarily directed against dsRNA, TLR7 and TLR8 recognizes ssRNA and TLR9 recognizes unmethylated CpG DNA. Indeed, numerous studies support this view. For example, TLR3 was shown to be critical in the recognition of dsRNA in endosomes in murine knock out experiments, where TLR3-/- mice had trouble clearing infections with dsRNA viruses (Alexopoulou, et al. 2001). TLR9 was first identified as a PRR for bacterial unmethylated CpG dinucleotides (Hemmi 2000). Later studies indicated a role for TLR9 in the innate immune activation upon DNA-virus infection (Lund, Sato 2003). Mice that were depleted of TLR9 expression gave higher virus titers after i.p. infection with the DNA murine herpes virus 68 (MHV-68). Additionally, TLR9 was shown to be involved in fighting off the infection in both lytic and latent (when nearly only DNA is present) phases (Gussemoos et al, 2007). TLR7 and TLR8 are associated with the induction of an innate response against ssRNA viruses, such as influenza and vesicular stomatitis virus (Xiao 2009). Thus, an important immune mechanism works via the recognition of a viral infection by endosomal TLRs via nucleic acid ligands.

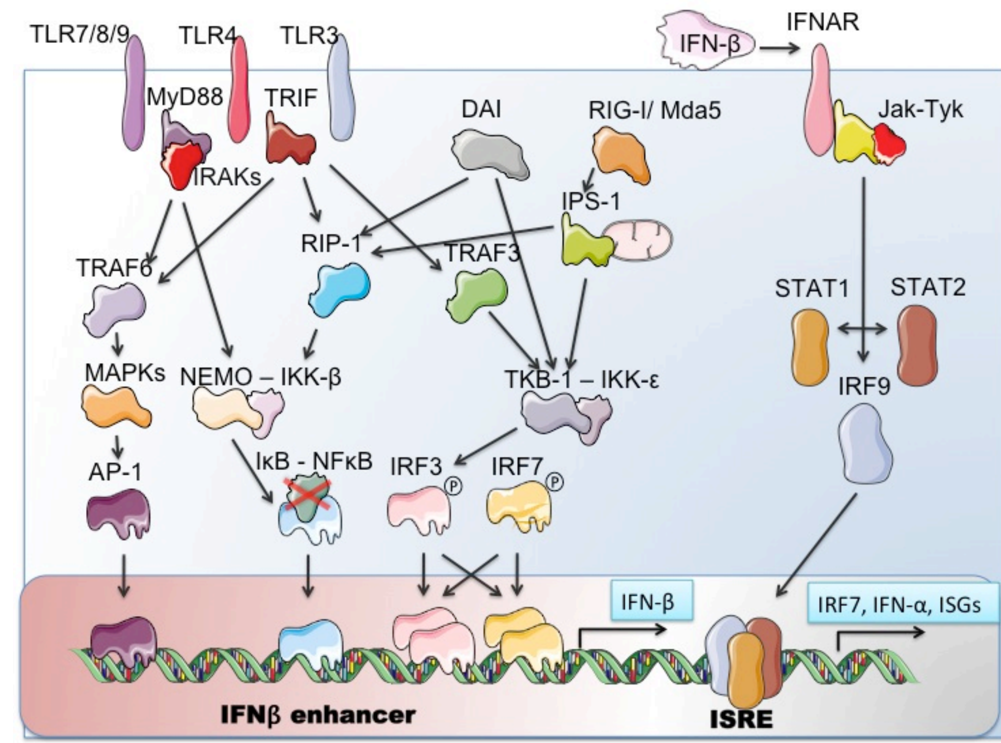

Figure 2. An overview of the signaling pathways that are important in the IFN type 1 response to viruses. TLR2, 3, 4, 7, 8, 9, DAI, and RIG-I/Mda-5 can recognize viruses. This recognition initiates signaling cascades through several adaptors that lead to activation of the transcription factors NF-κB, IRF3, IRF7, and/or AP-1 to transcribe several genes among which is IFN-β. A feedback loop through the IFNAR and Jak-STAT signaling initiates a broad and robust anti-viral immune state. RIP-1 and TKB-1 form integrating hubs in the meshwork of signaling cascades.

Signaling axes

One characteristic of all PPRs is their ability to activate three major signaling axes, of which a selection is initiated after recognition of viral compounds. The interferon regulatory factors (IRF), mitogen-activated protein kinases (MAPKs) and nuclear factor (NF)-κB pathways together evoke a robust antiviral response. Of these pathways, the IRF pathway is most important to anti-virus innate immunity.

TIR domain mediated signaling

TLRs invariably contain a cytoplasmic signaling domain; the Toll/ IL-1 receptor (TIR) domain. It mediates the initiation of downstream signaling by association with the TIR domain of downstream adaptors. Recognition of the TLR ligand leads to dimerization of the TLR, which in turn enables binding of the TIR-containing adapters. This event initiates the recruitment of a variety of downsteam adaptor molecules in the cascade. Five adaptors with TIR domains can be characterized in the signaling pathways downstream of TLRs. The most widely implemented adaptor molecule is named myeloid differentiation primary response gene 88 (MyD88) and mediates many signaling cascades (Doyle, et al. 2003). MyD88 interacts with the TIR domain of

all TLRs with the exception of TLR3. Deficiencies of this adaptor are causative for increased susceptibility for fungal, bacterial and viral infections. A second important adaptor protein in anti-viral immunity, the TIR containing adaptor-inducing IFN-β (TRIF), can be activated by TLR3 and also TLR4. [reviewed in (Kawai and Akira, Semin Immunol 2007)]. This pathway is independent of MyD88, has different targets and is of special interest as it signals into IRF3 mediated induction of IFN type 1. The TIR domain containing adaptor protein (TIRAP) is another adaptor downstream of TLR2 and TLR4 and is also of significance to anti-viral signaling. Thus after TLRs dimerizes, they bind their adaptors via a TIR-TIR domain. Through the down stream cascade, a semi-tailored innate response emerges.

Interferon induction

In order to elucidate an adequate response to viruses, the cell needs to adjust its transcriptome. Through evolution, the functioning of type I IFNs have proven to be an especially effective mechanism in fighting off viruses. IRFs, together with AP-1 and the high-mobility group protein HMG-I(Y) assemble the enhanceosome complex at the promoter region of IFN-1β, which leads to transcriptional activation. The IRF family of transcription regulators comprises nine members (IRF1-IRF9) and they all contain a defined DNA binding motif. As their name implies, they were first discovered to enhance the transcription of IFN type I genes. IRF1, IRF3, IRF5 and IRF7 interact with IFN response elements upon viral infection (Honda and Taniguchi 2006). The transcriptional activation of interferon stimulated genes (ISGs) directly and indirectly activates a plurality of anti-viral mechanisms.

IRF3

The stimulation-induced dimerization of TLR3 enables TRIF to bind the TIR domain of the receptor and subsequently form a complex with TRAF3 (Honda and Taniguchi 2006). This event leads to the activation of the inhibitor of NF-ϰB kinase etha (IKK-θ) and TANK (TRAF family member-associated NF-ϰB activator)-binding kinase-1 (TBK-1) and subsequently the activation of IRF-3 (Zhang, et al. 2007). IRF-3 is of extensive importance in viral infections, as thid transcription enhancer is a central player in the downstream signaling of anti-viral PRRs. It is constitutively expressed and needs to be phosphorylated at a serine residue in the C-terminal domain to be activated. This event enables IRF3 to dimerizes (either homodimerize of heterodimerize with IRF7) and subsequently localize to the nucleus where it associates with its targets. Generally, IRF3 is immediately activated after initiation of the stimulating pathway. This leads to the transcriptional activation of ISGs, mainly IFN-β. The first discovered direct suppressor of IRF3 activation is (human) Pin-1. This protein can recognize phosphorylated serine/threonine and alters the conformation of its ligand that leads to poly-ubiqutination and degradation of IRF3. This mechanism illustrates the natural way of inhibiting IRF3 (Saitoh et al,

2006).

Unlike IRF3, the expression of IRF7 is mainly mediated by Jak-STAT via IFN receptor ligand binding. The IFN-β that is produced after IRF3 gene enhancement, can bind to the INF-A receptor (IFNAR) in an autocrine and paracrine fashion. This initiates a signaling cascade that involves signal transducer and activator of transcription (STAT)1 and STAT2 and Janus kinase (Jak) and eventually lead to the hetrodimerization of STAT1 and STAT2 to form a complex with IRF9. This complex then locates to the nucleus where it functions as a transcription factor for ISGs and IRF7 by binding to IFN stimulating response element (ISRE) (Boo en Yang 2010). This ignites a robust innate immune response. Additionally, NF-ϰB signaling is induced by STAT activation. After this induction of IRF7 in pDCs, it is activated through an interaction with MyD88 and TRAF6 (Konno, et al. 2009). MyD88 was found to interact with IRF7 in the TLR7 and TLR9 initiated pathways and the feedback loop is the consequence of IRF3 activation (Kawai and Akira, Semin Immunol 2007). Like IRF3, phophorylation and dimerization precede its nuclear localization. IRF7 homodimers and heterodimers with IRF3 have differential effects on gene expression; IRF3 is a potent inducer of IFN1-β and IRF7 of INF-α. Thus, the IRF mediated pathway functions in a two-step manner, only evoking a robust response after a positive feedback loop inducing a wide range of IFN stimulated genes (ISGs). Additionally, also IRF1 and IRF5 were found to be downstream targets of the MyD88 pathway (Zhang, et al. 2007).

NF-ϰ B

Generally, two modes of activation in virus-infected cells are of significance in signaling NF-ϰB. The first starts with the direct activation of the adaptor MyD88. The second is due to the positive feedback loop that was described in the IRF pathway. Upon stimulation TLR7, TLR8, and TLR9 recruit and interact with MyD88. This event initiates the downstream signaling to members of the IL1 receptor associated kinase (IRAK) family of kinases is mediated by the death domain of MyD88. A complex is formed with IRAK-1, IRAK-4, and the catalytically inactive IRAK-2 and IRAK-M, which signals into the NF-ϰB and MAPK pathways (Ringwood, 2007). This initiates the polyubiqutination and degradation of IϰB form NF-ϰB. This event is required for the nuclear localization of NF-ϰB as it is retained in an inactive form, bound to IϰB by default. Typically, this leads to the expression of inflammatory chemokines and cytokines, such as IL-6, IL-12 and tumor necrosis factor α (TNF-α) as well as IFN-β (Kawai and Akira, Ann N Y Acad Sci 2008).

Crossroads in NF-ϰB and IRF signaling

The adaptor receptor-interaction protein kinase (RIP)-1 fulfills a centric role in various stress-induces signaling pathways triggered by death receptors and (importantly) pathogen recognition. It is one of the main links between the NF-ϰB and the IRF mediated pathways. The RIP1 RIG-I-

like helicase domain enables it to transduce a signal from activated RLRs to these pathways. It also has a RIP homotypic interaction motif (RHIM), which interacts with the RHIM domains of TRIF proteins that are important in IRF3 activation.

RIG-I like Receptors (RIG-I, Mda and LGP2)

Cytosolic PAMPs

Several viruses avoid the localization of viral nucleic acids to intracellular vesicular structures. They localize directly or mainly to the cytoplasm, revealing (nucleic acid) PAMPs in this compartment. The appearance of dsRNA in the cytoplasm is a clearly a non-physiological conditions. Synthesis of intermediate genome products by ssRNA viruses, genomes of dsRNA viruses and stem loop RNA generated by DNA viruses give rise to cytosolic dsRNA. Additionally, ssRNA that was abnormally processed indicate viral infections. Together, this forms a useful pool of PAMP for recognition by the second PRR family.

RLR domains and their function

The cytosolic counterpart of TLR family is referred to as retinoic acid-inducible gen-I (RIG-I)-like receptor (RLR) family and comprises RIG-I, melanoma differentiation factor-5 (Mda-5) and laboratory of genetics and physiology-2 (LGP2). RIG-I contains four domains, but Mda-5 and LGP2 lack the C-terminal and the two N-terminal caspase recruiting domain (CARD-like) domains, respectively. The CARD-like domain is responsible for eliciting the cascade by binding downstream targets and appears in duplo in the RLRs. The DExD/H box RNA helicase domain was found to possess ATPase activity, which is indisputable for signaling. The C-terminal domain is the primary RNA binding site. This fourth domain is referred to as the repressor domain (RD). By default, the RLRs are inactive because the RD physically blocks the CARD-like domain, whose accessibility is necessary for activation. However, as a result of ligand binding by the RNA-binding subdomain, this inhibition is released by ATP-dependent conformational changes. This subdomain is also present in Mda-5. This enables the CARD-like domains to bind their adaptors, hence start a signaling cascade [reviewed in (Xiao 2009)]. A mutation in RIG-I that deletes the RD will render a constitutively active RLR system (Kawai and Akira, Nat Immunol 2006). Thus, the RLR family of cytosolic receptors are auto-inhibitory by default, but are activated for adaptor binding upon (nucleic acid) ligand recognition.

The link between RLR ligand recognition and downstream effects remained elusive until the discovery of the adaptor protein interferon β promoter stimulator-1 (IPS-1). This adaptor contributed importantly to the understanding of the RLR mediated immunity. IPS-1 contains a CARD-like domain that is very similar to the RLR CARD-like domains. It was indicated to interact with the activation-exposed CARD-like domains of RIG-I and Mda-5. This linked RLR

activation to downstream signaling pathways (Johnson and Gale 2006). Furthermore, IPS-1 contains a transmembrane domain with which it anchors to mitochondria initiated by a sorting signal. Deletion of this domain attenuates the signaling capacity, indicating that the site of assembly is in some respect important for the function of the signaling-complex. Thus, IPS-1 is the main adaptor in RLR signaling, most similar to MyD88 in the TLR signaling.

IRF3 induction by RLRs

The inducible genes of RLRs also signal toward the induction of IFNs and ISGs. After association and activation of RLRs with IPS-1, the latter protein can interact with TRAF3 in complex with IKKθ and TKB-1. This interaction, as in the TLR3 initiated activation, enables downstream signal transduction, resulting in the phosphorylation of IRF3. So, the RIG-I and TLR3 pathways converge at TRAF3. Moreover, IPS-1 can also interact with RIP-1 connecting the RLR signaling with the other TLR signaling, terminating in NF-κB (Kawai and Akira, Ann N Y Acad Sci 2008).

Specificity of RLRs

The domain constitution of the three RLRs would indicate that there are differences between their functioning. Indeed, the lack of CARD-like domains in LGP2 implies that it is unable to start a signaling cascade via IPS-1. Initially, LGP2 was identified as negative regulator of RIG-I as a dominant negative agonist, while another study indicates that it stimulates Mda-5 signaling by hetero-dimerization (Venkataraman, et al. 2007). RIG-I and Mda-5 are less distinct as they both start a signaling cascade upon recognition of RNA through the ATPase and CARD-like domain. RIG-I has a profound preference for short dsRNA with a 5' triphosphate and without a cap, whereas Mda-5 is activated upon recognition of longer dsRNA molecules (Xiao 2009). Recently is has also been suggested that short and long ssRNA is also recognized by RIG-I and Mda-5, respectively. A wide variety of viruses are recognized by RIG-I, while Mda-5 seems only to be involved in picornaviruses and noroviruses recognition (Rasmussen, et al. 2009). This difference in specificity is thus far unaccounted for.

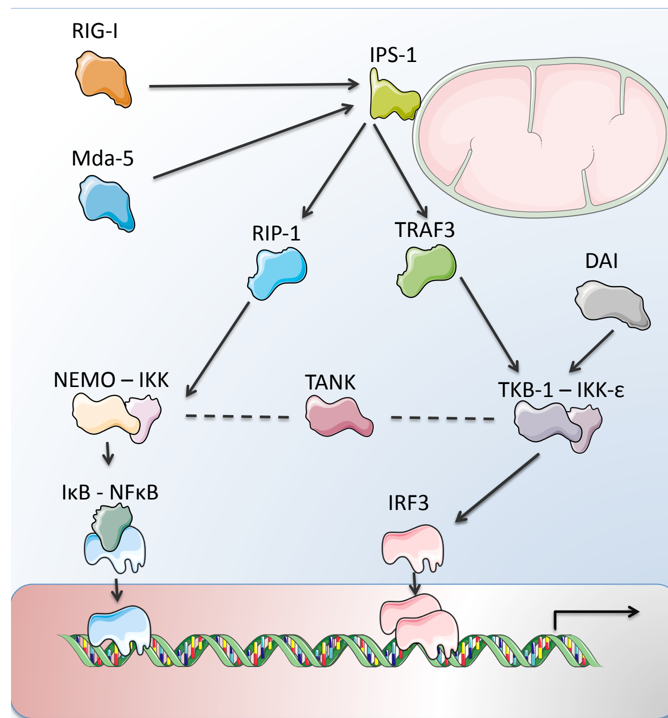

Figure 3. Intracellular PRR signaling. RIG-I and Mda-5 signal through the adaptor IPS-1 that is anchored in the membrane of mitochondria. Via RIP-1 and TRAF3 this signal can activate both NF-κB and IRF3, respectively. Also the DNA sensor DAI can activate both transcription factors via TKB-1 and TANK.

Other DNA sensors

Cytosolic RNA is a potent inducer of innate intracellular immune activation. However, it was only recently appreciated that also cytoplasmic DNA has this ability. This general principal reveals an important pool of intracellular PAMPs. The appreciation that replication of enveloped viruses is not required for DNA-induced immune signaling revealed a different type of innate recognition system; DNA sensors (Kawai and Akira, Ann N Y Acad Sci 2008).

Cytosolic DNA as PAMPs

Previously, the recognition of ribonucleic acids has been discussed because it is a potent PAMP in virtually all viral infections. However, this thesis discusses the immune evasion of herpesviruses, which are DNA viruses. Therefore, another class of nucleic acids (i.e. DNA) that in the recent years have been proven to be potent PAMPs, also need consideration. TLR9 was de first identified PRR to recognize DNA. However, several other mechanisms have been indicated to evoke an immune response after a DNA challenge. The DNA dependent activator of IRFs (DAI) (Takaoka, et al. 2007), absent in melanoma (AIM) (Hornung, et al. 2009) and recently also RIG-I and DNA dependent RNA polymerase (DdRd) III were postulated to have a role in DNA sensing (Choi, et al. 2009)(Chiu, Macmillan and Chen 2009).

An important issue when immune responses that are initiated by DNA sensors are considered is the chance on auto-immunity. When the DNA of the host triggers one of these sensors (in the wrong spacio-temporal situation) unnecessary auto-induced damage can occur. Tight regulation of the expression, localization and activation of DNA sensors is therefore evolutionary beneficial.

DAI

An interesting DNA sensor is the DNA-dependent activator of IFN-regulatory factors (DAI, also known as DLM-1/ZBP1). It selectively enhances the induction of IFN genes upon DNA stimulation. DAI can bind various types of DNA. It was initially discovered to bind DNA in a Z-conformation, but subsequently also B-DNA, synthetic-, and viral DNA. The N-terminus is necessary for the DNA-binding activity; this suggests that this DNA sensor needs sustained ligand association in its function (Takaoka, et al. 2007). The recognition of dsDNA by DAI induces a conformational change that enables the phosphorylation of DAI. Only when phosphorylated, DAI can associate with TBK1 and subsequently stimulate IRF3 activation (Wang, et al. 2009). The C-terminal domain of DAI is involved in protein-protein interactions with the IϰB kinase that controls the phosphorylation-dependent activation of IRF3 (Kaiser, Upton en Mocarski 2008). RIP1 is also the downstream target of DAI, thereby coupling this DNA sensing pathway to NF-ϰB. DAI interacts with RIP1 via its RHIM domain (Kaiser, Upton en Mocarski 2008).

DdRp III as a DNA recognition receptor

A recent paper (Choi, et al. 2009) provided evidence for DNA-recognition and signaling by RIG-I. IFNAR knock out MEF cells that were transfected with RIG-I induced ISG enhancement, whereas non-transfected did not. Moreover, RIG-I showed direct binding to B-DNA in a pull-down assay. In the same study, the MEFs were infected with HSV, which gave an ambivalent result, probably due to the several evasive strategies that are displayed by herpesviruses. Opposing or redundant to this finding was the appreciation that cytosolic poly (dA-dT) B-DNA can be converted into 5'-ppp RNA, which is the conventional ligand of RIG-I, by the DdRp III (Chiu, Macmillan and Chen 2009). These observations categorize DdRp III as an innate DNA sensor protein that triggers IFN production. Interestingly, the recent discovery of the RIG-I adaptor stimulator of IFN genes (STING) further links RIG-I to DNA-induced immune activation. The ablation of STING diminished the responsiveness to transfected B-DNA and to HSV-1 infection, compared to the control situation (Ishikawa and Barber 2008).

AIM2 as a DNA recognition receptor

A DNA sensor that is involved in the recognition of viral DNA by a rather different mechanism that those previously discussed is AIM2. Like DAI, it surveys the cytoplasm of foreign DNA,

however it induces the formation of an inflammasome after activation. This complex controls the catalytic cleavage of IL1β and IL18 in a caspase-1 dependent fashion (Hornung, et al. 2009). IL1β in particular is a cytokine that induces an overall inflammatory reaction. Its signaling stimulates, in addition to activating adaptive immune cells, the NF-κB pathway and thereby amplifying and broadening the immune response.

Protection

Herpesviruses have a DNA genome, the containment of (m)RNA in their tegument and their contiguous transcription in common. These traits provide recognizable ligands for PRRs. TLR2 and TLR4 could recognize their exterior, TLR3 and Mda-5 their hybridized two-directional transcripts, TLR7/8 and RIG-I could be activated by their ssRNA for the moment the virus enters the cell, and TLR9, DAI and other DNA-sensors could recognize their genome. Their pathways intertwine to be redundant, but also disperse to be broad.

Anti-viral mechanisms

After a primary IRF3 and IRF7 induced onset of ISGs a variety of autonomous mechanisms are initiated, but the mechanisms that are really able to handle an infection are not yet activated. IFNβ1 that is expressed as an early ISG, activates the IFNAR-STAT positive feedback circuit that activates more that 300 ISGs altogether. Many of them, such as 2'5'-oligoadenylate synthetase (OAS) and ribonuclease L (RNaseL), IFN-inducible dsRNA-dependent protein kinase (PKR), adenosine deaminase RNA-specific (ADAR) and apolipoprotein B mRNA-editing enzyme, and myxovirus resistance (Mx) protein interfere with the lifecycle of viruses (Bowie and Unterholzner 2008).

PKR is a RNA binding protein and is further activated upon ligand binding. When it encounters dsRNA autophosphorylation and homodimerization occur. This event activates the kinase domain of PKR, enabling it to phosphorylate and thereby inactivate eIF2α (Garcia, 2007). The RNA binding protein OAS also inhibits translation, after it is activated. anIt does so by destabilizing RNA globally. Otherwise, in the presence of its RNA ligand, OAS can be activated to catalyze the oligomerization of ATP into 2',5'-linked oligoadenylate, which in turn can activate RNase L. Activated RNase L degrades RNA of both viral and host origin. So, both PKR and OAS-RNase L are IFN induced proteins that shut down the cellular machinery in self-sacrifice to minimize viral spread (Ren Jye Lin, 2009). A recent report suggested a feedback mechanism as a critical component of type I IFN induction involving RNase L. The cleaved products of RNase L were suggested to be ligands for RIG-I activation and hence IFN induction (Rasmussen, et al. 2009). ADAR shares a RNA binding domain with RNase L and, like apolopoprotein B mRNA-editing enzyme, modifies the normal structure of viral RNA. The Mx protein has GTPase activity and a

leucine zipper with which it can recognize viral nucleocapsid and then bind to it to retain it to the cytoplasm or disrupt the assembly of virus particles.

Thus far, the cellular processes that fight off viruses have been described. PRRs recognize the presence of a virus infection to start off various cascades that lead to the activation of several anti-viral mechanisms. Viruses tend to manipulate these processes by acting on host proteins that play crucial roles in the signaling of these cascades. In the next chapter, these mechanisms will be placed in a pragmatic frame by assessing these anti-viral pathways in a background of herpesvirus infections.

PART III IMMUNOLOGICAL CONTEXT OF HERPESVIRUS INFECTIONS

Cellular responses to herpesviruses

An infected cell has a broad range of innate effector mechanisms to act on their intruders. Often, these mechanisms will suffice in evoking an effective response to the invasive virus. Although herpesviruses can be recognized by most of the cellular PPR, they manage to persist in latency meaning that they are able to evade the immune system. On the other hand, several mechanisms are able to respond to these viruses and evoke a (sub)effective anti-viral state.

HSV-1 and HVS-2

As is the case in all viral infections, type I INFs play a pinoval role in combating HSV-1 infection. The rigorousness and magnitude of INF expression negatively correlates to the viral titer in mice and patients. Indeed, HSV-1 infection initiates an IFN type 1 response (Nicholl, 2000). First evidence for a pathway that is triggered by HSV-1 to lead to IFN type 1 induction came from a TLR9 study. The deletion of TLR9 or its downstream signaling protein MyD88 lead to a diminished IFN-α and cytokine production in some cell types, but not in others. Moreover, although it was previously appreciated that HSV-1 DNA is a potent stimulator of an innate immune response (i.e. IFN expression), the presence of viral DNA alone did not induce protection (Melchjorsen, et al. 2006). This indicated that the archetypical DNA-PRR TLR9 is not on its own sufficient for protection. Another study showed a cellular response after viral transcription started in a TLR9 independent manner (Rasmussen 2007). Transcription of HVS-1 is necessary for cytokine expression in vitro in macrophages and DCs. This indicates that RNA-detecting PRRs are also important. Indeed, evidence for involvement of RIG-I and TLR3 was found in several studies (Rasmussen, et al. 2009)(Paladino and Mossman 2009). It was reported that dsRNA accumulates in HSV-infected cells (Weber et al., 2006), providing a ligand pool for RIG-I and TLR3.

In a study on the involvement of both TLR9 and RLRs in IFN induction upon HSV-2 infection, it was demonstrated that a combined activation of these PRRs is necessary for an adequate activation of innate signaling (Rasmussen, et al. 2009). Infected MEF cells with abrogated IPS-1 showed severely diminished IFN levels, compared to wild type MEFs. This was also the case in MEFs that were pre-treated with a specific antagonist to TLR9. When both PRRs were knocked down/out, no activation of IRF3 or NF-κB occurred, indicating that both pathways are functionally suppressing HSV-2 infections. Accordingly, TRIF/ MyD88 double knock out MEFs show no induction of IFN after TLR9 or RLR stimulation. However, the overlap was not complete as RLR -/- cells did show activation of the MAPK isoform p38 but not NF-κB and vice

versa, TLR9 antagonist induced activation of p38 but not NF-ϰB (Rasmussen, et al. 2009). In another study, TLR ligands were admitted onto a confluent monolayer of genital epithelial cells. The TLR3 and TLR9 ligands induced an anti-viral state to protect against HVS-2 infection, through stimulation of IFN type 1 and nitric oxygen species (NOS) (Nazli, et al. 2009). This finding indicates the importance of TLR3 and TLR9, and their downstream signaling, in the induction of an antiviral state against HSV-2, *in vitro*. Thus, several PPRs have been shown to be involved in combating HSV-1 and/or HSV-2, while the spatial-temporal aspects of the infection decide the outcome and effectiveness.

HCMV

TLR2 and DAI are probably the PRRs that together have the best immune activating capacity in cells that are infected with CMV (HCMV or murine CMV) (Power, 2008). UV-irradiated HCMV is able to induce IFNβ production, although not as rigorous as wt virus. This indicates that TLRs recognize virus particles (Browne, 2001), most notably TLR2 recognizing glycoprotein B. However, there are intracellular PRRs involved in CMV recognition as well. During HCMV infections IRF3 is phosphorylated and hence activated. DAI was found to be able to facilitate the activation of IRF3 and downstream targets upon HCMV infection (DeFilippis en Früh 2005). Additionally, TLR9 induction was shown to be required and necessary for the production of IFN-α in HCMV infections (Kawai en Akira, Nat Immunol 2006). Other experiments showed that an infection is inhibited by ligands for TLR2 and TLR9. This effect was IFN mediated as it was undone by administration of anti-bodies that are directed against IFN-1β (Harwani, et al. 2007). Although CMV compromises the responsiveness to viral infections, usually the induction of IFN-β is obverted, indicting an induction of both NF-ϰB and IRF3 transcriptional activation.

EBV and KSHV

The exact involvement of the plurality of PPRs in counter-acting infections by EBV and HKSV is less well studied. However, as is the case with HSV infections, IFN expression is an observed effect. Mouse studies of the murine γ-herpesvirus (MHV-68), which is closely related to EBV and KSHV, showed that TLR9 plays an important and organ specific role in the protection against this virus (Stevenson, Simas and Efstathiou 2009). The deletion of TLR9 decreased the production of IL-6, IFN-β, and IFN-α in DC cultures of infected mice (Gussemoos, 2006), which is the cause of an observed inhibition of the NF-ϰB pathway. The discovery of cytosolic DNA-sensors revealed a suitable set of PRRs for the recognition of γ-herpesviruses, however little research is done on this subject thus far.

Biopsy-derived Kaposi sarcoma tumor cells reacted to administration of synthetic RNA in a TLR3 dependent way and a reaction seizes to occur in TLR3 knockdown, using siRNA. An

opposite effect was observed for RIG-I (Livengood, 2007). KSHV was shown to specifically upregulate the expression of TLR3 in infected monocytes (both cultured and purified) in primary infections. UV-inactivated virions did not have this trait.

Little is known about the PRRs that are utilized in the detection and induction of anti-viral mechanisms by cells that are infected with EBV. However, TLR2 was found to recognize the dUTPase EBV protein and then actively induce the NF-κB signaling cascade, via MyD88 (Ariza, et al. 2009). Indeed, the transfection of dominant negative MyD88 mutants to a cell line that was subsequently infected with EBV, showed diminished expression of IL-6 and IL-10 (Ariza, et al. 2009). Moreover, EBV has an additional PAMP; the Epstein-Barr virus-encoded RNA (EBER) is a noncoding RNA that forms dimeric stem-loop structures, hence has a dsRNA-like appearance. It was proposed that EBER is actively exported out of infected cells, as it was found in substantial quantities in the sera of IM patients, where it associated with a cellular protein Lupus erythematosis-associated antigen (La). Active secretion as well as release from lysed cells enables EBER to be taken up by surrounding cells to induce TLR3 signaling (Iwakiri et al, 2009). Additionally, it can be bound by PKR and RIG-I. The induction of RIG-I signaling initiated by EBER was indicated by transient expression of RIG-I and EBER in EBV positive and negative Burkitt's lymphoma cells. It was shown that EBER induces IFN-β via the RIG-I pathway (Samanta, et al. 2006).

Discrepancy between immune effectors and their effect

Although various studies have underlined the potential of the intracellular innate immune system to cope with herpesvirus infections, human hosts are unable to ban these viruses from their system. This phenomenon can be explained by two herpesvirus characteristics; (i) the herpesvirus latency program and (ii) their extensively (co-)evolved countermeasures that target the innate immune pathways and molecules. Although the prior cause is a fascinating subject, it is hard to study. The latter characteristic of herpesviruses is of more interest here and can explain the discrepancies in the hypothetical and observable viral clearance that were discussed in PART I and the previous chapter of this PART. The next PART will discuss the various mechanisms that are used by several human herpesviruses to counter the host innate immune responses.

PART IV INNATE INTRACELLULAR IMMUNE EVASION OF HERPESVIRUSES

A considerable amount of viruses encode proteins that inhibit the innate immune response of host cells. Different viruses use different approaches. However, the focus of the evasive strategies of Herpes viruses is on a limited set of key pathways. The first discovery of viral inhibition of the pathways that activate IFN type 1 was in vaccinia virus (VACV). The VACV protein A46R that contains a TIR domain antagonizes the function of MyD88-containing signaling complexes. This finding gave a clue of the importance of IFN type 1 as anti-viral proteins and thereby opened the field for newly discovered inhibition mechanisms. Subsequently, the VACV protein A52R and the hepatitis C virus (HCV) protein NS3-4A where found to also inhibit IFN type 1 production (Bowie and Unterholzner 2008). Also RNA viruses target these pathways. For instance, de-ubiquitination by the nsp3 protein of murine hepatitis virus (MHV)-A59 inhibits the localization of IRF3 to the nucleus (Zheng, 2008). Many other viral proteins with similar functions (but often different mechanisms) were found.

Large DNA viruses use several strategies to avert an efficient innate immune response. Evasion in the latent phase evolves mostly around camouflage and keeping under the immunological radar. Indeed, bioinformatics studies show that herpesviruses have significantly less viral epitopes that could be expected from their (genome) size, and that their epitopes resemble host epitopes better than viruses without a latent phase. However, in the acute and lytic phase these viruses need to dampen the immune response in a direct fashion. Inhibition of host and viral protein degradation, induction of anti-apoptotic activities, shedding of a wide range of misleading viral chemokines, and mimicry of host regulators are all principals that herpes viruses apply to establish a permanent residence in the host. One of the most effective mechanisms is by inhibiting IFN induction.

Herpes viruses suppress IFN responses

The pathways that lead to the induction of IFN and IFN-mediated anti-viral mechanisms, as described in the first PART of this thesis, have several hubs and indispensible proteins that are especially suitable targets for viruses. Millions of years of co-evolution equipped herpesviruses with a broad arsenal of anti-IFN mechanisms, directed to such proteins.

The archetypical α-herpesvirus HSV-1 has six well-defined proteins that inhibit IFN anti-viral responses. These immunomodulatory proteins function in different stages of infection. Most of them have several functions that peek in one of the phases of infection. In the (immediate) early

phase Us3 suppresses the transcript level of TLR3, while the three viral proteins ICP0, ICP34.5, and ICP27 influence the normal function of IRF3. An archetypical β-herpesvirus (HCMV) also invests a considerable amount of its elaborate genome to the evasion of host immune mechanisms. Three proteins, viz. IE86, IE72 and UL83 are clearly pointed toward IFN elimination and two others, IRS-1 and TRS-1, are hypothesized to inhibit PKR function (which is an effector of IFN type 1 activity). The newly appreciated M45 protein seemed to inhibit RIP-1 functioning. KSHV has its own class of anti-IFN proteins that mimic host IRFs. Additionally, the two KSHV proteins encoded on ORF45 and ORF50 also negatively influence IFN induction. Finally, the mechanisms displayed by EBV are mediated by BGLF4 and LF2 as was recently discovered.

Targeting IRF3 and/ or NF-ϰB

As became apparent, the induction of the IFN transcription activator IRF3 is a critical event in the expression of IFN-β of which the signaling in turn is an important event of the initiation phase of the anti-viral cellular response. Therefore it is not surprising that all of the discussed herpesviruses encode one or more proteins that intervene in the normal induction of IRF3 by anti-viral PRRs (i.e. TLR3/ 7-8/ 9, DAI, or RIG-I).

The notion that a mutant strain of HSV-1, with an ICP0 deletion, is much more pathogenic in IRF3 knock out mice than in wt mice (Härle, et al. 2002), suggests that ICP0 has an effect on IRF3 mediated anti-viral response. A co-infection study with Sendai virus showed degradation and less nuclear localization of IRF3, providing evidence of a direct interaction. Later, the co-localization of ICP0 and IRF3 in the nucleus was shown in a single infection experiment (Melroe, DeLuca en Knipe 2004). This hinted to a mechanism in which IRF3 is sequestered by ICP0, making it unable to bind to its enhancer site. However, the mechanism by which ICP0 influences the function of IRF3 is still slightly ambiguous, as another study reported reduced phophorylation of IRF3 in the precence of ICP0 (Paladino and Mossman 2009). In contrast to this, a new study that was conducted with ICP0 transfections that was similar to the expression level of that of endogenously infected cells in the immediate early phase, showed that ICP0 is not able to inhibit IRF3 mediated gene expression (Everett and Orr 2009). A difference in multiplicity of infection or cell type could explain this discrepancy. The exact mechanisms remain to be unambiguously elucidated. The second HSV-1 protein that is involved in IRF3 inhibition is ICP27. A mutation-study revealed a role of this immediate early protein in the inhibition of NF-ϰB as well as IRF3. Transcriptional activity was much higher in macrophages that were infected with a ICP27 knock-out mutant compared to wt virus (Melchjorsen, et al. 2006). Surely this effect is dependent on the cell type (macrophages versus epithelial cells) (Paladino and Mossman 2009). When a third protein (ICP34.5) is expressed in mammalian cells, it inhibits IFN gene activation (Verpooten, et

al. 2009). However, the involved mechanism has long been not elucidated. A function of ICP34.5 of which the importance was only recently appreciated is the ability to inhibit TBK-1, which is a downstream signaling protein of DNA-recognizing PRRs (Paladino and Mossman 2009). ICP34.5 binds TBK-1 in the TIR domain thereby blocking it from IRF3 binding.

The HCMV tegument protein pp65 (UL83) interferes with the nuclear localization of either NF-κB, of IRF3 or both (Miller-Kittrell and Sparer 2009). This indicates a mechanism for immediate suppression of an IFN type 1 response in infected cells. In one *in vitro* infection study that was conducted with a pp65-deleted virus, the IFN response was higher that in the wt. Additionally, a transfection with UL83 alone was enough to block nuclear localization of IRF3 (Abate et al 2004). Other studies that used an adenovirus vector contradict these results. They found IRF1 and NF-κB to be induced in the mutated virus compared to the wt virus (Marshall and Geballe 2009). Both studies found consensus in the observation that UL83 inhibits nuclear localization of its target transcription factor. The exact mechanism is as yet unclear.

A recent study reported on the ability of macrophages to respond to bacterial challenge in the presence of an infection of either HCMV of EBV (Lin, 2009). In both assays, TLR9 expression levels and TNF-α production, which is a measure for NF-κB induction, were lower in the presence of these viruses. This indicates that both viruses use a direct or indirect mechanism to inhibit the pathway that terminates in NF-κB transcriptional activation.

The KSHV has developed its own strategy to inhibit the IFN induced anti-viral response. The KSHV genome encodes a cluster of four genes (vIRF1-4) that are viral homologues of IRFs. Thus far there are no vIRF described in HSV, HCMV or EBV. The vIRF1-3 were shown to antagonize the function of IRF3 and/ or IRF7 as dominant-negative. Only vIRF1 has the tryptophan residues in the N terminus of the protein that is required for DNA binding. It acts in the nucleus and interacts with p300 to inhibit transcriptional activation after IRF3-promoter association. Complementarily, vIRF2 inhibits IRF3 transcriptional activation through a different mechanism. It induces caspase-3 mediated decay of IRF3, without being pro-apoptotic (Areste, 2009).

One of the viral suppressors of the innate immune activation in EBV infected cells is the kinase BGLF4. This protein binds to IRF3 independent of the activation state of its target. This binding suppresses the promotional activation of IFN. Experiments that determined the exact state of IRF3 that is targeted by BGLF4, i.e. phosphorylation, dimerization, relocation to the nucleus, or domain III binding, showed that the prior three steps are unaffected. Three phosphorylation sites in IRF3 seem to be important for the functioning of BGLF4 as deletions of these sites dampen the

attenuating effect of the kinase. Thus, physical interaction and phosphorylation of IRF3 inhibit binding of the IRF-response element of the IFN type I promoter (Wang, et al. 2009).

Targeting IRF7

While IRF3 is the key facilitator of the immediate signaling and gene induction upon PPRs viral activation, IRF7 is usually the prime amplification element in the IFN cascade. It sets the cell to a robust, well suited and broad anti-viral state. TLR9 can directly activate IRF7. Attenuating the normal function of IRF7 is therefore of essence to herpes (DNA) viruses.

Adding to the putative inference with IRF3, the viral protein ICP0 has a plurality of other functions in countering the onset of antiviral mechanisms. The protein domain that was identified to be most important is the RING finger domain, which has ubiquitin ligase (E3) activity. Beside IRF3 inhibition, ICP0 is involved in IRF7 inhibition, degradation of the protein promyelocitic leukemia (PML) leading to disassociation of nuclear P-bodies, STAT1 activation and RNase L independent RNA degradation. As ICP0 is expressed fairly late during infection, these effects mainly take place in the transition from the latent to the lytic phase (Paladino and Mossman 2009). The exact mechanisms of these immune evasive effector functions are not clearly understood.

Also vIRF3 (KSHV), which is also known as latency associated nuclear antigen (LANA)-2, has an effect on IRF7 rather than on IRF3 (in contrast to what the name would imply). Binding of vIRF3 to the DNA-binding domain of IRF7 ensures the loss of gene enhancement (Joo, et al. 2007)(Lee, et al. 2007). Additionally, the gene products of the two KSHV genes ORF45 and ORF50 both specifically inhibit IRF7. ORF45 binds to it and makes it unable to be phosphorylated (Xiu Zhu, 2002). The ORF50 gene product replication and transcription activator (RTA) also binds to IRF7, but its mechanism involves ubiquination-induced proteosomal degradation (Coscoy 2007).

The EBV tegument protein LF2 was recently shown to have a suppressive effect on innate immune activation. This was shown in a study that screened the effect of several EBV proteins on the transcriptional activation of IFN-α genes in a luciferase reporter approach, after Sendai virus infection that is known to induce of type I IFN. LF2 diminished the activation of IFN-α1, -4 and -6 promoters, which are regulated primarily by IRF-7. A clue for a mechanism of this inhibition came from the observation that LF2 binds to IRF7. Further competition precipitation assays indicated that LF2 inhibits the dimerisation of IRF-7, which is required for its activation (Wu, et al. 2009). So, LF2 inhibits the second phase IFN/ ISG induction by binding IRF7, prevent dimerization and therefore inhibit localization to the nucleus.

The latently expressed KSHV protein vFLIP has a promoting activity on NF-ϰB signaling by binding to TRAF family members. Interestingly, it is necessary for KSHV survival. This notion emphasizes the delicate balance of cells and the activation of immune effector molecules. For instance, the NF-ϰB pathway is also involved in cell growth, which can provide the virus with an expanding reservoir or an easy excess to the nucleus. A benefit for the host (i.e. the intended clearance of the virus) can easily turn into the induction of a non-beneficial persistence (reviewed in Coscoy, 2007).

Targeting the IFNAR and Jak-STAT signaling

Another function was found for ICP27 in inhibiting STAT1 signaling (Johnson and Gale 2006). STAT-1 nuclear localization was observed in ICP27 deletion mutant HSV-1, but not in wt infections indicating an involvement in the IFN induced amplification of the anti-viral response via STAT-1. However, the primary mechanism of ICP27 immune modulation is in its ability to modulate host transcripts (Paladino and Mossman 2009).

Although the cytomegalovirus has one of the widest ranges of immuno-modulation strategies, it has only a few genes that are known to act on the expression of IFNs and ISGs. The two immediate early HCMV genes IE86 and IE72 have been shown to inhibit IFN expression. The IE86 interacts with the IFNβ promoter site to prevent NF-κB from binding (Marshall and Geballe 2009). IFNR, STAT1 and STAT2 transcript levels are all diminished in CMV infected cells. IE72 influences the IFN induced immune amplification loop by preventing STAT2 to bind its targets (Miller-Kittrell and Sparer 2009)(Paulus 2006). After the first signaling cascade that is induced by PRR recognition of viral PAMPs, with IFN-β as an important product, an additional positive feedback cascade is induced through IFN-A receptor activation. The Jak-STAT downstream signaling is inhibited by the viral protein RIF (ORF10). This protein forms an inhibitory complex with the cytosolic domain of IFNAR and the signaling proteins Jak1 and Tyk2. It also recruits STAT2 to the complex in an abnormal fashion. Together this leads to an impairment of IFNAR signaling, and hence a dampened immune response.

Also EBV abrogates the Jak-STAT signaling. The two EBV proteins LMP2A and LMP2B accelerate the turnover of the IFNAR. These latent proteins can thereby lower the responsiveness of the cell to exterior signals. They do so by a process that enhances the acidification of endosomes. This marks the vesicles as lysosomes and activates several lytic enzymes.

Targeting IFN-induced effector proteins

The classical function of ICP34.5 in the inhibition of host defense mechanisms is the reversion of PKR activity (Chou, 2002). ICP34.5 binds to the protein phosphatase 1α that is activated to dephosphorylate the elongation factor eLF2b, hence to prolongate protein synthesis. The HCMV

proteins IRS1 and TRS1 have dsRNA binding activity with as yet unspecified implications. However, the mouse CMV encodes two proteins, with dsRNA binding capacity, that are involved in the suppression of PKR activity most likely by competition for the RNA ligand.

The HSV-1 proteins Us11 and vhs also negatively influence the immune response through IRF independent ways. The late gene Us11 (although it is also abundant in the tegument) targets OAS and PKR. The mechanism by which Us11 attenuate the activity of OAS is not exactly clear, however it was suggested that Us11 sequesters RNA to protect it from catalytic alteration by OAS (Sànchez and Mohr 2007). Two studies have shown Us11 to bind to PKR and its protein activator (PACT) (Paladino and Mossman 2009). The tegument-carried late phase viral protein host-shutoff (vhs) interferes with the immune response in a more general way. It is long known to degrade viral and host mRNA. This means that also the amount of INF and ISG transcript is diminished by vhs, which dampens the immune response.

Another interesting immuno-modulation is induces by a HCMV viral protein that mimics the immunosuppressive chemokine IL10. In the host, this cytokine suppresses inflammatory cytokine expression and interferes with MCH class II presentation. The CMV virus, like other large DNA viruses, encode their own (v)IL10 to induce immune suppression by binding to the IL10 receptor (Miller-Kittrell and Sparer 2009) (Kotenko, 2000).