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Toll-like receptor-mediated recognition of herpes simplex virus

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1. ABSTRACT

Herpes simplex virus type 1 and 2 (HSV-1 and HSV-2, respectively) are two important human pathogens that belong to the genus simplex within the subfamily alpha of the *Herpesvirinae.* Toll-like receptors (TLRs) constitute a family of conserved sensors that play a prominent role during the early anti-viral response, including that against herpesviruses. Although substantial progress has been made, central questions remain to be solved to figure out how TLRs modulate viral pathogenesis. The aim of the present report is to review the current knowledge about TLR recognition and signaling of herpesviruses, focusing on HSV infection. The relative contribution of the TLR-mediated immune responses to antiviral immunity versus viral pathogenesis will be discussed as well.

2. INTRODUCTION

The host innate immune response is critical in providing the first line of defense against pathogens, including viruses. This response, although not antigen specific, is able to discriminate among a great variety of microorganisms. Early recognition of microorganisms occurs through a limited number of germ line-encoded pattern-recognition receptors (PRRs), which recognize pathogen associated molecular patterns (PAMPs). PAMPs are invariant motifs distinct from those of the host, which are essential for the pathogen to survive and thus are unlikely to be altered. Toll-like receptors (TLRs) form a highly conserved group of transmembrane PRRs that are able to recognize a wide range of PAMPs found on pathogens, playing a relevant role in the innate immune

Table 1. PAMPs recognized by human TLRs

PAMP	Organism	TLR involved	References
LPS[1]	Gram-negative bacteria	TLR4	10, 29, 33, 34, 87-89
Viral envelope glycoproteins	Viruses		
microbial components	Bacteria, mycobacteria, mycoplasma,fungi, protozoa	TLR2 (TLR1/TLR2 Or TLR6/TLR2)	35, 42-45, 58, 65, 68, 70, 90-93
Viral envelope glycoproteins	Viruses		
dsRNA	Viruses	TLR3	49, 86, 94-96
Flagellin	Bacteria	TLR5	97, 98
ssRNA	Viruses	TLR7 and TLR8	18, 19, 37, 96
CpG DNA	Bacteria, mycobacteria, viruses	TLR9	20-22, 28, 51, 54, 56, 57, 99, 100
Undetermined	Undetermined	TLR10	101

Abbreviations: [1]lipopolysaccharide

response (1-3). TLRs also play a pivotal role in the initiation of the adaptive immune response through the induction of cytokine and chemokine expression involved in leukocyte recruitment or direct T cell activation by TLR agonists (4-7). Each TLR has the ability to recognize different PAMPs derived from pathogens, including bacteria, fungi, protozoa and viruses. Viral glycoproteins and nucleic acids constitute the main viral PAMPs recognized by TLRs. A list of the moieties recognized by each TLR is presented in Table 1. The participation of TLRs in immune protection was first observed in *Drosophila,* where Toll, a protein involved in dorso-ventral patterning, was found to regulate the expression of the antifungal peptide drosomycin (8). One year later, the group of Charles Janeway, reported the cloning and characterization of a human homolog of *Drosophila* TLR, termed hToll (9). Soon afterwards, Poltorak *et al.*, identified loss-of-function mutations in the gene *tlr4*, coding for the mouse homolog of hToll, to be the cause for the hypo responsiveness of mice to bacterial lipopolysaccharide (LPS) (10). A significant portion of the TLR biology has been elucidated by studying this receptor, now termed TLR4. Since those pioneer studies came to light, 10 TLRs have been described in human, 9 of which are conserved in mouse (1, 3, 11).

Herpes simplex virus 1 and 2 (HSV-1 and -2, respectively) are important human pathogens. The prevalence of HSV-1 is estimated to be more than 90% and that of HSV-2 around 22% in the adult U.S. population, reaching up to 80% in sub-Saharan Africa and developing countries (12, 13). Primary infection with HSV takes place in the mucosa. In general, HSV-1 preferentially infects through the oro-labial mucosa and establishes latency in the trigeminal ganglia whereas HSV-2 does so through the genital mucosa, establishing latency in the sacral ganglia. However, both viruses can infect either mucosa. Disease caused by HSV is normally mild in immuno-competent individuals. However, under certain circumstances, HSV infection can have devastating consequences. Thus, HSV-1 infection of the cornea can cause stromal keratitis lesions, termed herpes simplex keratitis (HSK), that represent the most common cause of infectious blindness. Moreover, HSV infection of the central nervous system (CNS) can lead to herpes simplex encephalitis (HSE), an outcome more often observed in non-immunocompromised patients. Infection of the neonate with either HSV-1 or -2 may be limited to the skin, eye or mouth but it can also affect the CNS. Furthermore, HSV disseminated infection in neonates involves the lung, liver and the adrenal gland. These symptoms resemble those characteristics of bacterial sepsis. Finally, several reports suggest a synergistic effect of HSV-2 on the infection by human immunodeficiency virus (HIV) and progression towards acquired immunodeficiency syndrome (14, 15).

The host immune system contains several mechanisms to counteract herpesvirus infection. Despite the existence of an organized and orchestrated immune response, HSV and other herpesviruses are able to persist in the organism during the lifetime of the individual. HSV, like other herpesviruses, contains several PAMPs that are recognized by TLRs, resulting in the expression of cytokines and chemokines with antiviral activity. Cells targeted by HSV, both in the mucosa and the CNS, express TLRs. The context of the interaction between the different TLRs and HSV might determine the outcome of HSV infection, including the clearance or spread of the virus and the absence or presence of disease. In this regard, recent data suggest that HSV recognition by certain TLRs may play a role in HSV-mediated pathogenesis. Finally, several groups are investigating the use of TLR agonists to inhibit HSV replication and progression to disease.

3. INDUCTION OF AN ANTIVIRAL RESPONSE BY TLRS

3.1. Basis of TLR structure and signaling

The structure of TLRs resembles that of the IL-1 receptor (IL-1R). TLRs consist of an N-terminal extracellular domain containing variable leucine-rich-repeat (LRR) motifs, followed by a transmembrane region and a C-terminal cytoplasmic signaling domain homologous to that of IL-1R, and hence called the Toll/IL-1R homology (TIR) domain. Ligand binding to the receptor induces its dimerization, and causes a series of conformational changes that lead to the recruitment to the TIR domain of TIR-domain-containing proteins. There are four adaptor proteins bound by TIR: Myeloid differentiation primary response protein 88 (MyD88), TIR-associated protein/MyD88-adaptor-like (TIRAP/MAL), TIR-domain-containing adaptor protein-inducing interferon-beta/TIR-domain-containing molecule 1 (TRIF/TICAM1) and TRIF-related adaptor molecule (TRAM). The core of TLR signaling relies on MyD88. MyD88 is used by all TLRs with the exception of TLR3, which signals exclusively through TRIF (1, 3), and TLR4, which is able to signal through both MyD88 and TRIF, the latter in combination with TRAM (16). The use of different

Table 2. Herpesviruses recognized by TLRs

Subfamily	herpesvirus	Viral PAMP	TLR involved	references
alpha	HSV[1]	Unknown viral protein dsRNA CpG motifs	TLR2 TLR3 TLR9	56-58, 65, 68, 70 49, 86, 96 18, 52-56, 63
	VZV[2]	Unknown viral protein	TLR2	44
beta	CMV[3]	gB and gH glycoproteins dsRNA CpG motifs	TLR2 TLR3 TLR9	42, 43 22 22, 28
gamma	EBV[4]	Unknown viral protein	TLR2	45
	KSHV[5]	Envelope protein	TLR4	29

Abbreviations: [1]Herpes simplex virus, [2]varicella-zoster virus, [3]cytomegalovirus, [4]Epstein-Barr virus, [5]Kaposi sarcoma herpesvirus

combinations of adaptors by each TLR permits the initiation of different responses by a given TLR.

Signaling through the MyD88-dependent pathway results primarily in the activation of nuclear factor kappa B (NF-kappaB) and mitogen activated protein kinases (MAPK), inducing the expression of proinflammatory chemokines and cytokines. The TRIF-dependent pathway mainly activates NF-kappaB and interferon responsive factor 3 (IRF3). Overall, either signaling pathway ultimately results in the expression of cytokines with antiviral activity such as interleukin (IL-) 6, IL-12, tumor necrosis factor-alpha (TNF-alpha) and interferon (IFN). Most studies showed type I IFN expression upon stimulation with TLR3, TLR4, TLR7, TLR8 and TLR9 (1, 3, 17-24). Recently, induction of IFN-gamma in T helper 1 effector cells by TLR2 ligands was described (5). Prompt expression of this immune mediator is essential to control viral replication and therefore many mechanisms work in a cell-type and time-dependent manner to trigger IFN response. A detailed description of TLR signaling is beyond the scope of this report; however, a number of interesting reviews concerning different aspects of TLR signaling are available (1, 2, 11, 25).

Most TLRs are found on the cell surface, except for TLR 3, 7, 8 and 9, that are localized in endosomes (1). The relative importance of the subcellular localization of these receptors in the generation of anti-viral responses has not been fully clarified yet. Compartmentalization of TLR and TLR-mediated expression of co-stimulatory signals in dendritic cells (DCs) allows discrimination of non-self from self ligands (26). Intracellular localization is required to favor TLR and viral nucleic acid interactions. Endosomal localization may limit access to "self" nucleic acids (18, 19, 27). Thus, targeting of TLR9 to the plasma membrane abrogated TLR9-mediated recognition of viral nucleic acids whereas it allowed recognition of self nucleic acids (27). On the other hand, TLR4, TLR2/TLR1 and TLR2/TLR6 recognize viral glycoproteins and are expressed on the cell surface (1-3, 11).

Although the first TLRs discovered in mammals were identified due to their anti-bacterial role, the prominent role of TLRs in the generation of an early immune response against viruses became clear in the last few years (18, 20, 22, 28-31). The discovery that vaccinia virus expressed two proteins (A46R and A52R) that suppressed TLR signaling strongly suggested that TLRs could play a role in the antiviral response (32). This was soon confirmed with the first evidence for the recognition of the fusion protein of respiratory syncytial virus (RSV) by TLR4 (33). Since then, a role for several TLRs in the antiviral response has been established. Together with RSV, mouse mammary tumor virus (MMTV)-induced activation of TLR4 (34), while measles virus (MV) stimulated the production of cytokines such as IL-6 in a TLR2-dependent manner (35). Additionally, the ability of MMTV to activate cells also through TLR2 has been proposed (36). Initial studies on TLR7 and TLR8 implicated them in the response to RNA viruses, such as HIV, influenza virus and vesicular stomatitis virus (VSV) (19, 37). TLRs recognize viral proteins or viral nucleic acids in the form of ssRNA, dsRNA or unmethylated CpG motifs present within the viral genomes. As shown in table 1, TLR3 recognizes dsRNA, TLR7 and 8 ssRNA and TLR9 unmethylated CpG motifs. Viral proteins, in turn, are recognized by TLR2, which forms a heterodimer with TLR1 and TLR6, or by TLR4 (1, 3, 11). For excellent reviews on the complex interplay between viruses and the TLR system see Boehme and Compton (25) or Finberg *et al.* (38).

4. THE INTERPLAY BETWEEN HERPESVIRUSES AND TLRS

Herpesviruses turn on a complex innate immune response, which includes IFN production, multiple cytokine synthesis and the secretion of chemokines that recruit and activate inflammatory cells (39-41). Nevertheless, not all the mechanisms used by the immune system to trigger the inflammatory response against these viruses are fully understood. The TLR network constitutes one of the main players in the early detection and response against herpesviruses. Recent data demonstrated the recognition of different human pathogens of this family by TLRs (Table 2). Compton *et al.* showed for the first time the ability of human cytomegalovirus (hCMV) to trigger inflammatory cytokine production through CD14- and TLR2-dependent activation of NF-kappaB (42). Later on, this group identified two envelope glycoproteins of hCMV that were implicated in TLR2 engagement (43). The impairment of TLR3 and TLR9 signaling, in turn, had a dramatic effect on the progression of murine CMV-induced pathogenesis (22). More recently, the recognition of Varicella-Zoster virus (VZV) by TLR2 in human monocytes was described (44). The work of Gaudreault and colleagues showed that infectious and UV-inactivated Epstein-Barr virus (EBV) virions activated NF-kappaB specifically through TLR2. Furthermore, EBV infection of human monocytes induced the release of the monocyte chemotactic protein 1 (MCP-1), and such chemokine response was significantly reduced after treatment with small interfering RNA (siRNA)

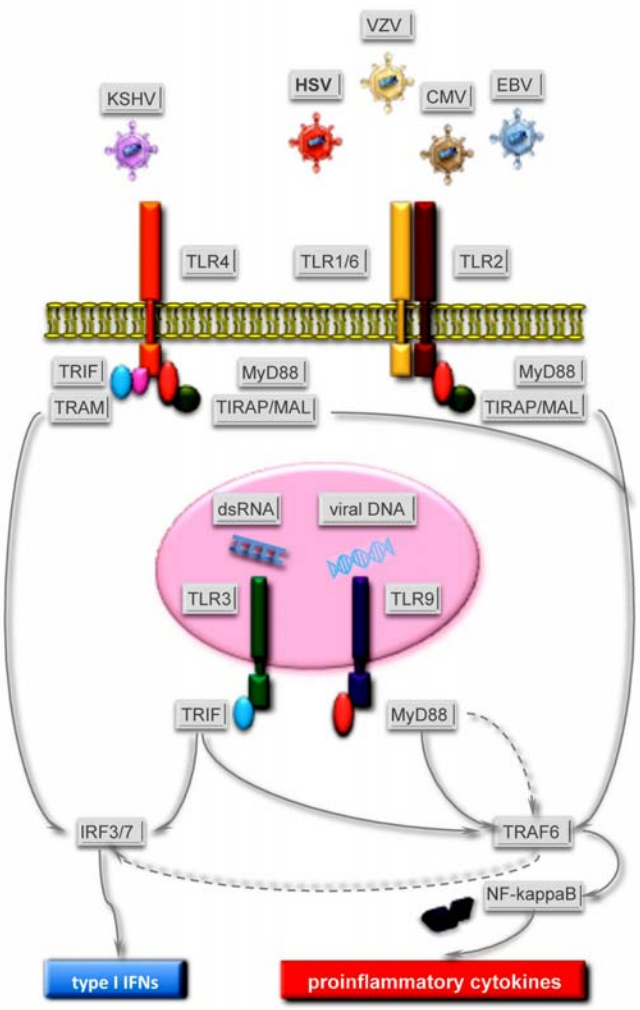

Figure 1. Induction of cytokine expression upon TLR-dependent recognition of herpesviruses. TLR-mediated recognition of herpesviruses is essential for the correct development of an antiviral response, and for the coordination of the adaptative immunity. Members from all subfamilies within the *Herpesviridae* family activate TLRs, triggering a potent immune response. TLR2, present at the cell surface as a dimer with TLR1 or TLR6, recognizes HSV, VZV, CMV and EBV. TLR4, also found at the plasma membrane, interacts with the gammaherpesvirus KSHV. HSV and CMV engage both TLR3 and TLR9 expressed within the endosomal compartment, via recognition of dsRNA and CpG motifs present on the viral genome respectively. All TLRs contain a cytosolic signaling motif (TIR domain) where specific downstream adapters (MyD88, TIRAP/MAL, TRIF) and signaling transducing proteins are recruited. MyD88 is used by all TLRs, with the exception of TLR3 that signals exclusively through TRIF, and TLR4 that is able to act through MyD88 or TRIF (in combination with TRAM). TLR signaling through MyD88 pathway leads to the activation and nuclear translocation of NF-kappaB, inducing proinflammatory cytokine and chemokine production. TRIF-dependent pathway activation upon stimulation of TLR4 or TLR3 results in inflammatory cytokine expression via NF-kappaB as well as in type I IFN production through IRF activation. In pDCs, stimulation of TLR9 upon viral DNA recognition, causes the expression of high levels of IFN and cytokines through a MyD88- and IRF7-dependent mechanism (discontinuous line).

targeting TLR2 (45). The second human gamma-herpesvirus, Kaposi's sarcoma-associated herpesvirus (KSHV), the causative agent of KS, is the first herpesvirus reported to be detected by TLR4 (29). The antiviral activity of TLR4 had been previously demonstrated mainly against RNA viruses, such as RSV (33).

As mentioned previously, herpesviruses are able to persist lifelong in the infected host despite the immune response triggered against them. This indicates that herpesviruses have evolved strategies to modulate and evade the immune system, including the TLR network. For instance, EBV manipulates the TLR7 pathway for its own benefit (46). This pathway provides signals that drive naïve B cells, the target of EBV infection, to proliferate. Thus, it was suggested that during the earlier steps of infection, EBV manipulates TLR7 signaling to promote the initial phase of B-cell activation and expansion. However, later during infection, the virus induces negative regulators of the TLR7 pathway, necessary for the establishment of

latency (46). Another example of a herpesvirus capable of modulating the TLR system can be found in KSHV. A rapid drop of TLR4 expression was reported during KSHV primary infection of endothelial cells (29). Macrophages lacking the *tlr4* gene and cells treated with anti TLR4 siRNA, appear to be more susceptible to KSHV infection, since impaired cytokine production and higher viral gene expression is observed (29). Thus, the authors suggest that the downregulation of TLR4 could be a mechanism employed by the virus to escape the control of the immune system. The fact that a mutation in a *tlr4* allele predisposes KSHV infected individuals to suffer from multicentric Castleman's disease, a lymphoproliferative disease associated with KSHV, is an additional piece of evidence for the involvement of TLR4 in the innate immunity against the virus (29). Moreover, KSHV seems to induce TLR3 signaling during primary infection (47). As a result of TLR3 activation, a series of cytokines and chemokines that act as potent chemoattractans and angiogenic factors are produced (47). The authors speculate that the modulation of TLR3 signaling by KSHV aids the virus to spread and to establish latency before the acquired immune response starts (47).

4.1. Recognition of HSV by TLRs

The oro-labial, genital mucosa and the cornea constitute the initial sites of HSV infection. Following local replication, HSV reaches a peripheral nerve and thereby the trigeminal or sacra ganglia where latency is established. In some instances, HSV infects the CNS and other organs. Cells targeted by HSV in the mucosa, the cornea and the CNS express TLRs that recognize and trigger an immune response against HSV. HSV displays PAMPs recognized by several members of the TLR network, and the role of such interactions is yet to be totally defined. Experiments carried out using knockout mouse models lacking either one or two TLRs, or MyD88 suggest that innate resistance to HSV is achieved by the regulated activation of several TLRs (21, 30, 31, 48, 49). In fact, HSV is recognized by at least TLR2, TLR3 and TLR9 and experiments carried out both *in vitro* and *in vivo* support the notion of an orchestrated response against HSV (21, 30, 31, 48, 49). It is well established that PAMP recognition by means of the TLRs expressed in DCs is required for the induction of T cell responses just after an infection (2). DCs present a distinct TLR expression profile depending on the cell subtype. In humans, plasmacytoid DCs (pDCs), also termed IFN producing cells (IPCs), only express TLR7 and TLR9 (50, 51). Upon infection, pDCs express high levels of all subtypes of type I IFN. IFN induces the expression of cytokines involved in monocyte maturation, antigen cross-presentation to T-cells, leukocyte differentiation and activation (50-52). This process seems to be independent of viral replication, since UV-inactivated HSV triggered identical IFN production (21). HSV contains a great amount of unmethylated CpG motifs in its genome, that serve as ligands for TLR9 (53). In fact, pDCs produce extremely high amounts of type I IFN following recognition of HSV-2 via TLR9 (20, 21, 51). However, mice lacking TLR9 can still control HSV replication in a cutaneous infection model, suggesting that TLR9-independent mechanisms can compensate the impaired pDC response observed in these animals (20). Hochrein *et al.*, using pDCs isolated from different tissues of wt and *Tlr9*$^{-/-}$ or *Myd88*$^{-/-}$ mice, showed that IFN-alpha response after HSV infection was generated by TLR9-dependent and independent mechanisms, and mediated by different cell types other than pDCs (54). Thus, fibroblasts, macrophages and other non-pDCs have been shown to produce IFN-alpha in response to HSV infection in a TLR9 or MyD88 independent manner, but dependent on viral entry and replication (55). In contrast, HSV-induced expression of other cytokines, such as TNF and CCL5 by macrophages is mainly dependent on TLR9 (55). Thus, the existence of two waves of type I IFN production during HSV infection has been suggested (55). The synthesis of IFN-alpha/beta shortly after infection would be performed by pDCs mainly in a TLR9-dependent manner, whereas the subsequent IFN production would be mediated by other cell types using mainly TLR9-independent mechanisms (55).

The existence of synergy between TLR2 and TLR9 was observed using single knockout mice for either receptor or double knockout mice (56). Following HSV-2 infection, there were no major differences regarding cytokine response and viral load between wt and *Tlr2*$^{-/-}$ or *Tlr9*$^{-/-}$ (56). Importantly, the *Tlr2*$^{-/-}$/*Tlr9*$^{-/-}$ mice had impaired cytokine production and higher viral loads in the brain but not in the liver (56). Interestingly, a recent report described a previously uncharacterized mechanism of viral detection, that functions through the sequential recognition of HSV by TLR2 followed by TLR9, within the same DC (57). This serial activation of multiple TLRs in a given cell, could represent an evolutionary trick that allows the immune system to more precisely identify a pathogen, in order to mount the optimal response against it (57). Monocyte/macrophages constitute another cellular compartment infected by HSV that plays a relevant role in HSV recognition and control (58). Infection of macrophages by HSV results in the production of IFN-alpha/beta, TNF-alpha and chemokines through different signaling pathways (55). TLR2 mediates IL-15 expression upon HSV-1 infection of human monocytes (58). Inhibition of TLR9 signaling impaired TNF-alpha expression whereas IFN-alpha/beta was not affected (58). HSV infection affects TLR signaling through mechanisms that are not fully understood. One of them seems to occur through the interaction between HSV ICP0 and ubiquitin specific protease 7 (USP7). ICP0 induces the translocation of USP7 to the cytoplasm where it deubiquitinates the TNF-associated factor (TRAF)-6 and the inhibitor of kappa B kinase (IKK)-gamma thereby abrogating TLR-mediated NF-kappaB and JKK activation (59). Moreover, HSV-1 infection of monocytes *in vitro* downregulated TLR2, TLR4, and monocyte activation markers such as CD38 and CD69 whereas it increased the presence of necrosis and apoptosis markers (60). Atopic dermatitis patients are particularly susceptible to HSV infection. Pro-inflammatory monocytes obtained from these patients showed affected TLR2-mediated TNF-alpha and IL-1beta production (61).

The main TLR playing an anti-HSV role in the CNS is TLR3. This TLR recognizes dsRNA produced

during HSV infection and has been shown to control HSV-1 infection, particularly in the CNS. Studies carried out *in vitro* using human monocyte cell lines suggest that HSV US3 controls TLR3-mediated signaling against the virus at least in this context (62). The importance of TLR3 in HSV-mediated pathogenesis is supported by the findings that a dominant-negative point mutation in this receptor may predispose to HSE (49). Monocyte-derived DCs, $CD8^+$ T cells, natural killer (NK) cells and fibroblasts obtained from patients carrying this mutation are impaired in IFN production upon stimulation with polyinosinic:polycytidylic acid (poly I:C), a ligand of TLR3, and are not able to control HSV-1 or VSV infection (49). The importance of TLR signaling in HSE is supported by the finding that mutations in UNC-93B, a protein required for the signaling of TLR3, 7, 8, and 9, correlate with a higher prevalence of HSE in humans (63).

4.2. Immunopathological consequences of TLR activation by HSV

Many of the symptoms observed in the pathogenesis associated with herpesviruses are linked to the presence of inflammatory and angiogenic factors. Several herpesviruses encode chemokines and chemokine receptor homologs that provide strong inflammatory signals and induce angiogenesis (64). These viral homologs of cytokines recruit inflammatory cells that in turn may transport the virus to other potential sites of infection, or can serve for the establishment of virus latency. According to the majority of mouse models of infection used to date, the signals transmitted by PRRs are essential for pathogen clearance and host survival. However, several reports suggest that herpesviruses are able to modulate the TLR network, suggesting a role for TLR signaling in herpesvirus-mediated immunopathogenesis.

Regarding HSV, several reports indicate the involvement of the TLR system in the immunopathogenesis observed. The study by Kurt-Jones demonstrated that TLR2 recognition of HSV-1 mediates an excessive inflammatory response, which was associated with viral encephalitis rather than with a protective response (65). Animal experiments carried out by this group using wt and *Tlr2*$^{-/-}$ mice, showed that TLR2 mediates the induction of multiple proinflammatory cytokines and chemokines in response to HSV (65). The attenuated cytokine response found in *Tlr2*$^{-/-}$ mice correlated with absence of brain inflammation, and was followed by an important reduction of the mortality rate, compared to wt or *Tlr4*$^{-/-}$ mice (65). Importantly, the differences observed in *Tlr2*$^{-/-}$ mice were not due to viral titers, which were equivalent to those found in wt animals. Since the same trend was observed in neonatal animals, the authors proposed that neonate susceptibility could be due to an excessive TLR2-dependent response, rather than being associated with the inability of their immature immune system to contain the infection. Indeed, the production of higher levels of IL-6 and IL-8 by peripheral blood mononuclear cells (PBMCs) isolated from human neonates, compared to that from adults, has been reported in response to the challenge with HSV-1 or HSV-2 (66). A similar detrimental response is observed during certain bacterial infections in neonates, particularly to those antigens that activate TLR2 (67). Aravalli *et al.*, using microglial cells isolated from wt and *Tlr2*$^{-/-}$ mice, reported the requirement of this receptor for the production of many proinflammatory cytokines and chemokines in response to HSV infection (68). Microglial cells are known to play a key role in neuroimmune responses, once they are TLR stimulated (69). The implication of TLR2 signaling in the induction of apoptosis in HSV-infected microglial cells has been suggested (70). These examples point towards a potential role for TLR2-dependent signaling in HSV-induced neuropathology. Additionally, a recent report found an association between two TLR2 haplotypes and an increase in shedding and lesional rates in HSV-2 infected patients. Therefore, polymorphisms in this receptor may be in part responsible for the observed differences in the severity of HSV infections in humans (71). A role for TLRs on HSK has been proposed (31, 72). Using a corneal scarification disease model, the pathological assessment of MyD88 and TLR knockout mice was examined. Mice lacking MyD88 do not suffer from such pathology but die from encephalitis, indicating the role played by TLR signaling in the immunopathology observed in wt mice and in control of virus spread (31). Deletion of single TLRs pointed to a role of TLR2 and, to a lesser extent, TLR9 in the onset of keratitis. Interestingly, mice lacking TLR4 had more pronounced lesions than wt mice (31). Corneas suffering from active HSK express higher levels of TLR mRNA than healthy corneas, in particular those of TLR4, 7, 8, and 9, whereas TLR7 was the sole upregulated TLR in non-active HSK (72). Thus, TLR2, TLR4, 8 and 9 seem to be involved in the pathogenesis observed in active HSK (31, 72).

Taken together, the results presented here provide strong evidence that TLR-mediated responses, in particular those mediated by TLR2, play a prominent role in HSV-associated immunopathology. However, it is important to bear in mind that the context of the interaction between the different TLRs and HSV could determine the outcome of HSV infection. Factors such as age, immunological status and genetic background may have an impact on TLR-related immunopathogenesis.

5. POSSIBLE THERAPEUTIC IMPLICATIONS OF TLR AGONISTS AGAINST HSV-MEDIATED PATHOGENESIS

Due to the pivotal role of TLRs in the immune response several groups are investigating the potential use of TLR agonist as prophylactic or therapeutic agents. The observations presented above open the door to the use of TLR ligands as a promising approach to treat HSV infections in the clinic. Each year there are about 500,000 new cases of HSV-2 infection in the USA (13), and there is currently no vaccine available against the virus. Initial studies examined the use of TLR ligands as adjuvants in mucosal immunization strategies against HSV-2, and more recent reports suggested a protective effect of direct local delivery of these agents. Genital epithelial cells (GEC), which constitute the first line of mucosal defense, were shown to express mRNA for all TLRs, although at different levels (73, 74). A number of studies have shown the

efficacy of intra-vaginally (IVAG) delivery of TLR9 and TLR3 agonists to elicit potent innate immune responses dependent on IFN-beta against HSV-2 (73, 75-80). Furthermore, this protection correlated with the production of nitric oxide, IFN-beta and other cytokines, following addition of TLR3, TLR5 and TLR9 ligands to polarized GEC (73). The relevant role of IFN-beta was confirmed using knockout mice and by the IVAG administration of IFN-beta (77). The activation of TLR4 failed to provide any protection (73, 77), while the use of TLR2 ligands had no or little effect against HSV-2 infection (73, 77). Despite these reports indicating lack of efficiency when TLR2 agonists were used solely, immunization studies showed that fusion of a TLR2 agonist to a HSV-2 CD8 T cell epitope resulted in the recruitment of specific memory CD8 cytotoxic T cells both in the genital tract, the lymph nodes and spleen that conferred protection against HSV-2 challenge (81). The response was diminished when *Tlr2*$^{-/-}$ and *Myd88*$^{-/-}$ mice were immunized, showing less HSV-2-specific T cell responses and higher viral titers, disease and death rates than wt mice (81).

The use of imiquimod and resiquimod, ligands with TLR7/8 mixed agonist activity, has been tested in preclinical and clinical studies. Although with varied success, these studies suggested the ability of these agonists to promote a Th1 specific response that controlled HSV-2 mucosal infection (82, 83). CpG oligodeoxynucleotide (CpG ODN), a TLR9 ligand, is more efficient than resiquimod (R-848, TLR7/8) when administered IVAG in mice and causes a local immune response against HSV-2 (84). The lVAG delivery of CpG ODN protects against IVAG HSV-2 challenge in mice (75-78), however this treatment is associated with local inflammation and splenomegaly (85). IVAG delivery of dsRNA, in turn, induced protection without those symptoms (76). Similarly, the TLR3 ligand, poly I:C, also protects against genital HSV-2 infection without apparent local immunotoxicity (79).

Regarding HSV-1, the use of a mouse model for HSE indicates that the activation of TLR3 through intranasal delivery of poly I:C triggers the expression of TLR3, TNF-alpha, IL-1beta, IL-12, IFN-gamma and CXCL10; reduces HSV loads and increases survival (86). Higher survival rates were also observed after treatment with a TLR9 agonist, compared to those of wt or TLR4$^{-/-}$ mice (86). The relevance of TLR3 in protection against HSE is supported by the finding that individuals with naturally-occurring mutations causing lack of TLR3 function, have higher susceptibility to suffer from lethal encephalitis as a result of HSV-1 infection (49).

Only the use of TLR agonists has been investigated so far. However an issue that arises from the works outlined in this review, is the possibility to use inhibitory compounds of certain TLRs to restrain the excessive antiviral inflammatory response that results harmful to the host.

6. CONCLUDING REMARKS

Our knowledge of the TLR-virus relationship has improved dramatically in the last few years. The list of TLR viral partners will expand as more TLR pathways are discovered in the years to come. Research on this field has only started to unravel how TLRs impact on virus-mediated pathogenesis. The finding that several herpesviruses belonging to different subfamilies, together with RSV, MMTV and measles virus, can activate innate responses in a TLR-dependent manner, confirms the main role played by the TLR system in antiviral immunity. Additional support comes from the observation that some viruses have evolved mechanisms to modify or counteract the TLR network.

HSV recognition by TLRs is essential for the initiation of a prompt innate immune response and the coordination of the adaptive immune response. TLR activation results in the secretion of high amounts of type I IFN and other cytokines such as IL-12, providing a link between innate and adaptive immunity. TLR3 stimulation, in turn, seems to reinforce an immune mechanism of neuroprotection against the virus (86). On the other hand, TLR-mediated responses can also have detrimental consequences for the host. This is clearly illustrated by the fact that TLR2 recognition of HSV in a particular context causes serious immunopathological lesions, responsible for a significant portion of the morbidity and mortality associated with the infection (31, 65). Therefore, defining the individual role of each TLR alone and in combination is crucial to understand how TLRs modulate pathogenesis. This is particularly interesting in the case of herpesviruses, to date, the only viral family known to interact with up to five TLRs.

Analysis of the molecular basis of TLR and viral PAMP interactions will be decisive in clarifying the cellular mechanisms by which the immune response is regulated. Understanding how TLRs and viruses interact will provide insight into our own immune system and into viral and host co-evolution, and will be of great help in developing more efficacious antiviral strategies.

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Abbreviations: HSV: Herpes simplex virus, TLR: Toll-like receptor, PRR: pattern-recognition receptor, PAMP: pathogen associated molecular pattern, LPS: lipopolysaccharide, HSK: herpes simplex keratitis, CNS: central nervous system, HSE: herpes simplex encephalitis, HIV: human immunodeficiency virus, IFN: interferon, IL: interleukin, IL-1R: IL-1 receptor, LRR: leucine-rich-repeat, TIR: Toll/IL-1R, MyD88: Myeloid differentiation primary response protein 88, TIRAP/MAL: TIR-associated protein/MyD88-adaptor-like, TRIF/TICAM1: TIR-domain-containing adaptor protein-inducing IFN-beta/TIR-domain-containing molecule 1, TRAM: TRIF-related adaptor molecule, NF-kappaB: nuclear factor kappa B, MAPK: mitogen activated protein kinase, IRF3: interferon responsive factor 3, TNF-alpha: tumor necrosis factor-alpha, DC: dendritic cell, RSV: respiratory syncytial virus, MMTV: mouse mammary tumor virus, MV: measles virus, VSV: vesicular stomatitis virus, CMV: cytomegalovirus, VZV: Varicella-Zoster virus, EBV: Epstein-Barr virus, MCP-1: monocyte chemotactic protein 1, siRNA: small interfering RNA, KSHV: Kaposi's sarcoma-associated herpesvirus, pDC: plasmacytoid dendritic cell, IPCs: IFN producing cells, USP7: ubiquitin specific protease 7, TRAF: TNF-associated factor, IKK: inhibitor of kappa B kinase, NK: natural killer, PBMC: peripheral blood mononuclear cell, GEC: genital epithelial cell, IVAG: intra-vaginally, CpG ODN: CpG oligodeoxynucleotide, poly(I:C):polyinosinic:polycytidylic acid

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