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HIGHLIGHT

Innate immune exploitation by a model herpesvirus

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Host innate immunity represents the first line of defense against invading pathogens and shapes the course and outcome of pathogen infection. Mammals have evolved an array of highly conserved pattern recognition receptors (PRRs) that monitor the presence of "non-self" components or danger signals (Akira et al., 2006; Medzhitov, 2007). The innate immune signal transduction and viral regulation have been extensively reviewed elsewhere (Zhang et al., 2010), we therefore briefly summarize the signaling cascades that upregulate the transcription of antiviral inflammatory cytokines in response to viral infection.

In higher eukaryotes, PRRs are classified into three categories according to their structural and functional conservation, including membrane-anchored C-type lectin, toll-like receptors (TLRs), and cytosolic NOD-like or RIG-I-like receptors (NLRs and RLRs). Upon infection, PRRs sense pathogen-associated molecular patterns (PAMPs) that are usually pathogen structural components or replication intermediates (Akira et al., 2006; Medzhitov, 2007). While TLRs patrol extracellular and endosomal compartments, NLRs and RLRs monitor the cytosolic environment for foreign or self danger entities. Upon association with PAMPs, PRRs dimerize with their cognate adaptors (such as MyD88, TRIF, MAVS [also known as VISA, IPS-1, and CARDIF], or RIP2) that trigger the activation of two closely-related innate immune kinase complexes, IKKαβγ and TBK1-IKKε (also known as IKKi) (Sun et al., 2010). Through phosphorylation, these kinase complexes induce the activation of NFκB and interferon regulatory factors (IRFs), transcription factors that, in turn, upregulate the expression of antiviral pro-inflammatory cytokines and interferons (Chen et al., 1996; Mercurio et al., 1997; Fitzgerald et al., 2003; Sharma et al., 2003), thereby constituting potent antiviral innate immunity. The critical roles of these innate immune pathways have been elucidated by studies using gene knockout mice and conversely, investigations of pathogen evasion strategies that antagonize host innate immune responses. Within this short article, we will summarize our recent findings and introduce a new concept, innate immune exploitation whereby host innate immune activation is usurped by viruses to benefit their infection, for example, to promote viral replication.

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1 A model herpesvirus exploits a MAVS-dependent innate immune pathway to promote lytic replication

1.1 A proviral role of MAVS in γHV68 lytic replication

Herpesvirus is one of the most ubiquitous pathogens in humans. Hallmark of all herpesviruses is their remarkable propensity to establish a lifelong persistent infection in the presence of active immune responses, testimony of their successful co-evolution in contending with host immune system. Herpesvirus infection is linked to a variety of malignancies in immuno-compromised individuals such as AIDS patients or organ transplantation recipients. Of particular medical relevance are human gamma herpesviruses, including Kaposi's sarcoma-associated herpesvirus (KSHV) and Epstein-Barr virus (EBV), both of which are lymphotropic, tumorigenic DNA viruses (Boshoff and Weiss, 2001; Carbone et al., 2009). Due to a paucity of human studies and lack of permissive cell lines and animal models, host immune responses and *in vivo* infection of KSHV and EBV are largely uncharacterized, imposing a formidable obstacle for developing new antiviral therapeutics. The closely-related murine gamma herpesvirus 68 (γHV68) infects mice, replicates to high titers in the lung, and establishes persistent infection in the spleen, providing a tractable animal model to delineate the entire course of host responses and viral infection *in vivo* (Virgin et al., 1997). The genetically modified mouse strains and a bacterial artificial chromosome to generate

recombinant γHV68 constitute a powerful system to examine virus-host interactions. Thus, mouse infection with γHV68 provides a useful surrogate for human KSHV and EBV.

To investigate the roles of MAVS in gamma herpesvirus infection, we have examined γHV68 acute infection in the lung and latent infection in the spleen of wild-type and MAVS-deficient mouse. To our surprise, the loss of MAVS reduced viral load in the lung, while had no significant effect on γHV68 latent infection as determined by three independent measurements, including viral genome frequency, viral reactivation, and persistent infection in splenocytes (Dong et al., 2010). This remarkable phenotype was counter-intuitive according to the known antiviral activity of MAVS. The *in vivo* requirement of MAVS for efficient lytic replication of γHV68 was further substantiated by a number of observations derived from γHV68 infection in mouse embryonic fibroblasts (MEFs). Specifically, γHV68 lytic replication was delayed in MAVS-deficient MEFs than in wild-type MEFs as determined by multi-step growth curves. Consistent with this, γHV68 formed fewer plaques in MAVS-deficient MEFs than wild-type MEFs, indicating that MAVS is necessary for the initiation of γHV68 lytic replication. Moreover, the MAVS expression reconstituted by lentivirus in MAVS-deficient MEFs restored γHV68 lytic replication, supporting the specific requirement of MAVS. Given that the IKKαβγ-NFκB pathway and the TBK1-IKKε-IRF pathway relay signal downstream of MAVS, we took advantage of MEFs deficient in critical components of both pathways to genetically map the effectors downstream of MAVS that are necessary for efficient γHV68 lytic replication. This approach unambiguously identified IKKβ and IKKγ, but not the closely-related IKKα and components of the IRF-IFN pathway, whose deficiency recapitulated the replication defects of γHV68 in MAVS-deficient MEFs. Indeed, γHV68 infection transiently upregulated IKKβ kinase activity in wild-type MEFs that was otherwise quickly diminished in MAVS-deficient MEFs, suggesting that sustained IKKβ kinase activity requires MAVS during early γHV68 infection. Moreover, treatment with an IKKβ-specific inhibitor, Bay11-7082 (Pierce et al., 1997), prohibited γHV68 lytic replication by plaque assay in a dose-dependent manner. Again, the IKKβ expression reconstituted by lentivirus restored γHV68 lytic replication in IKKβ-deficient MEFs. Interestingly, exogenous expression of IKKβ in MAVS-deficient MEFs did not restore γHV68 lytic replication, suggesting that the MAVS-dependent activation of IKKβ, rather than the absolute amount of IKKβ, is critical for γHV68 lytic replication, highlighting the critical roles of MAVS-dependent innate immune signaling events in regulating IKKβ kinase activity during γHV68 infection. These findings collectively support the conclusion that MAVS and IKKβ are necessary for γHV68 lytic replication.

2 Molecular dissection of γHV68 lytic replication

To explore the molecular mechanism by which MAVS is used to promote γHV68 lytic replication, we examined the early events, namely viral entry into cells and viral transcription, by assessing levels of intracellular γHV68 genomic DNA and mRNAs in MAVS-deficient MEFs infected with γHV68. Results from these experiments indicate that comparable amount of γHV68 genomic DNA was found in wild-type and MAVS-deficient MEFs, supporting that γHV68 entry is normal in MAVS-deficient MEFs. Interestingly, levels of viral mRNAs, representing genes of immediate early, early, and late phases, were significantly reduced in MAVS-deficient MEFs than those in wild-type MEFs, suggesting that MAVS is required for viral gene transcription. Conversely, upregulation of IKKβ kinase activity by exogenous expression of TRAF6 greatly increased levels of viral mRNAs in a transient transfection system. These experiments entailing conditions of loss-of-function and gain-of-function pointed to the critical roles of MAVS and IKKβ in upregulating viral mRNA levels, likely through promoting viral gene transcription.

Notably, γHV68 encodes its own transcription factor, known as replication transcription activator (RTA). For γHV68 and KSHV, RTA is necessary for viral lytic replication and sufficient to reactivate productive replication in latently-infected cells. To further explore the hypothesis that the MAVS-IKKβ pathway is utilized to promote viral transcriptional activation, we tested whether IKKβ can directly phosphorylate RTA. Indeed, in vitro kinase assay demonstrated that purified IKKβ was capable of phosphorylating RTA within its carboxyl terminal transactivation domain. Radioactive labeling also revealed the MAVS- and IKKβ-dependent phosphorylation of RTA in γHV68-infected MEFs. Mutational analysis further identified two clusters of serine and threonine residues, i.e., STS and TTS, within the RTA transactivation domain. Mutations that replaced these two clusters of serines and threonines with alanine residues (designated STS/A or TTS/A) independently reduced RTA phosphorylation by IKKβ, with a more pronounced effect by the STS/A mutations. To assess the effect of IKKβ-mediated phosphorylation on RTA transcriptional activation, we performed reporter assays using viral lytic promoters that harbor RTA-responsive elements. In fact, exogenous expression of IKKβ increased RTA transcription activity by 1.5 fold. Remarkably, when RTA variants containing the STS/A or TTS/A mutations were used, IKKβ failed to increase RTA-dependent transcriptional activation. Moreover, the basal transcriptional activity of both RTA variants were lower than that of wild-type RTA by approximately 50%–80%, implying that endogenous IKKβ or other kinases phosphorylate RTA within these serine/threonine-rich clusters. Nevertheless, these results identified novel

phosphorylation elements within the RTA transactivation domain, linking viral gene transcriptional activation to the MAVS-dependent IKKβ kinase activated by γHV68 infection.

3 Recombinant γHV68 reveals critical roles of RTA phosphorylation in viral lytic replication

The bacterial artificial chromosome system provides an effective approach to introduce mutations into γHV68 and allows the functional interrogation of specific biochemically defined domains in viral infection. In this case, we generated recombinant γHV68 carrying mutations within the IKKβ phosphorylation sites, including the STS/A and TTS/A mutations. While the STS/A mutation abolished γHV68 lytic replication, we successfully obtained recombinant γHV68 containing the TTS/A mutation, along with a rescued recombinant γHV68 carrying wild-type RTA. The fact that the STS/A variant failed to support γHV68 lytic replication implies that RTA phosphorylation is critical for γHV68 infection possibly through, in addition to transcriptional activation imposed by IKKβ phosphorylation, other unknown mechanisms.

Using recombinant γHV68 carrying the TTS/A mutation, we have compared its lytic replication to the recombinant γHV68 rescued with wild-type RTA. When viral infectivity was normalized against intracellular viral genome copy numbers, the immediate early transcript of RTA was 32-fold higher expressed from wild-type γHV68 more than that of the recombinant γHV68.TTS/A, as determined by quantitative real-time PCR. This dramatic difference agrees with the notion that RTA auto-regulates its own expression during viral infection. Furthermore, the lytic replication of recombinant γHV68.TTS/A, was greatly impaired, producing viral titers that were less than 0.1% of that of recombinant γHV68 carrying wild-type RTA, when multi-step growth curves were determined by a low dose (0.01) of γHV68 infection in wild-type MEFs. Remarkably, the recombinant γHV68.TTS/A replicated with similar kinetics and produced similar viral titers in wild-type MEFs and MEFs deficient in MAVS or IKKβ, supporting the notion that MAVS, IKKβ, and RTA phosphorylation function within the same pathway to promote viral transcriptional activation. However, it was noted that mutations abolishing the IKKβ phosphorylation sites within RTA yielded much more pronounced effects on γHV68 lytic replication than those exerted by the loss of MAVS or IKKβ. Conceivably, additional kinases may phosphorylate RTA and promote its transcriptional activation. Taken together, our findings collectively support the conclusion that γHV68 infection activated the MAVS-dependent IKKβ to promote viral transcriptional activation and lytic replication, providing the first example whereby innate immune activation was coupled to viral gene transcriptional activation, thereby facilitating viral infection.

4 A paradox of IKKβ activation by γHV68 during lytic replication?

Although our data collectively demonstrated that IKKβ, activated by γHV68 infection, promoted viral transcriptional activation and lytic replication, activation of IKKβ and its downstream NFκB transcription factors potentially induces antiviral cytokine production. Moreover, RelA (also known as p65), a major subunit of the transcriptionally active NFκB dimer, was reported to inhibit γHV68 lytic gene transcription and viral replication (Brown et al., 2003). Investigation to solve this conundrum is necessary to further understand the interplay between gamma herpesviruses with the critical NFκB pathway.

5 Other related studies that support innate immune exploitation by viruses

5.1 Gamma herpesvirus exploits TLR-dependent signaling for latent infection

Although the loss of MAVS had no significant impact on γHV68 latent infection, MyD88-deficiency resulted in ca. 10-fold reduction in B cells harboring γHV68 genomes (Gargano et al., 2008), suggesting that the TLR-mediated innate immune signaling events are exploited by γHV68 to establish long-term latent infection. However, no defect of viral latency was observed in mice deficient in TLR3, a prominent PRR functions upstream of MyD88. These observations imply that multiple TLRs play redundant roles to signal via MyD88 in γHV68 latent infection. Alternatively, a TLR(s) other than TLR3 may be the primary PRR that engages MyD88 to promote γHV68 latency establishment (Gargano et al., 2009). Nevertheless, this intrinsic defect is, at least partly, due to reduced NFκB signaling, because a recombinant γHV68 expressing the dominant negative IκBα super-suppressor further reduced the frequency of γHV68 latently-infected B cells. Moreover, adopt transfer experiments demonstrated that MyD88-mediated signaling was required for γHV68-infected B cells to undergo class-switch and to differentiate in germinal centers (Gargano et al., 2008). This study indicate that MyD88-, and likely TLR-, dependent innate immune signaling is important for γHV68-induced B cell maturation and efficient establishment of γHV68 latency. In a parallel study, Gregory et al. (2009) reported that TLR signaling controls KSHV reactivation from latency. Specifically, natural and synthetic ligands for TLR7 and TLR8 activated viral lytic gene transcription and replication in latently infected primary effusion lymphoma cells. Moreover, knockdown of TLR8, but not TLR7, impaired

KSHV reactivation from latency, indicating the essential role of TLR8-dependent signaling in KSHV reactivation (Gregory et al., 2009). These findings implicated that, after primary infection, subsequent pathogen encounters with latently infected B cells can reactivate KSHV replication by TLR, although the molecular mechanism by which KSHV reactivation is linked to TLR-mediated signaling remains unknown. Given that the MyD88-dependent signaling converges with the MAVS-mediated signaling at the IKKαβγ and that RTA is important for KSHV reactivation, it is plausible that RTA phosphorylation by TLR-dependent IKK contributes to KSHV reactivation. The differential exploitation of MAVS and MyD88 in gamma herpesvirus lytic replication and latent infection, respectively, is consistent with the tissue distribution and functional relevance of these two signaling pathways relayed by the two adaptors. The molecular events of TLR- and MyD88-mediated innate immune signaling in gamma herpesvirus reactivation and latency establishment are waiting to be uncovered.

5.2 Human immunodeficiency virus (HIV) exploits innate immune signaling downstream TLRs and a C-type lectin

Innate immune cells such as dendritic cells (DCs) and macrophages are important reservoirs for HIV persistent infection (Cavert et al. 1997; Orenstein et al., 1997). DCs express a broad spectrum of PRRs of all three functional groups and, to date, both TLRs and C-type lectins are known to be involved in HIV recognition through diverse PAMPs. As such, HIV infection in DCs activated the NFκB pathway in a MyD88-dependent manner in which upstream TLR7 and TLR8 sense HIV single-stranded RNA (Heil et al., 2004; Beignon et al., 2005; Meier et al., 2007). Meanwhile, the HIV membrane glycoprotein 120 (gp120) engages DC-SIGN, a C-type lectin, and induces Raf-1 kinase activity that phosphorylates RelA. These signaling cascades eventually promote antiviral cytokine production that, in turn, either directly curtails viral replication or triggers adaptive immune responses.

Interestingly, it was discovered that HIV subverts TLR8- and DC-SIGN-mediated innate immune signaling to facilitate its replication in DCs and subsequent transmission to T cells (Gringhuis et al., 2010). Mechanistically, TLR8 signaling triggered NFκB activation that assists viral gene transcription initiation, while Raf-1 activation by DC-SIGN phosphorylates RelA that recruits pTEF-b, which subsequently phosphorylates RNA polymerase II to promote transcription elongation. Simultaneous innate immune signaling activation by TLR8 and DC-SIGN is necessary for productive replication of HIV in DCs, uncovering an intricate coordination between two innate immune signaling pathways that feed into the activation of NFκB transcription factor, RelA. Within this scenario, RelA has a dual role in HIV productive transcription, i.e., transcription initiation and elongation. It remains unknown how RelA in transcription initiation is biochemically different than that in transcription elongation. This information will shed light on functional specification of RelA conferred by post-translational modifications and/or associated factors in transcriptional regulation, a key step that we know very little about.

6 Concluding remarks

We have witnessed diverse strategies that pathogens deployed to evade and subvert host immune responses. These four recent studies have pointed to an important advancement of our understanding viral interactions with host immune system, i.e., pathogens can exploit host immune signaling for their benefit. Whether these immune exploitation strategies can be applied to diverse pathogens remain an open question. For example, have KSHV and EBV evolved similar tactics to usurp the MAVS-IKKβ pathway to promote viral transcriptional activation and lytic replication? Moreover, if these immune exploitation strategies are ubiquitously found, these findings will impinge on our definition of antiviral innate immune responses. In general, these findings will also instruct us on antiviral therapeutic design targeting relevant virus-host interactions.

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