The interferon-inducible DNA-sensor protein IFI16: a key player in the antiviral response

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—————————— SUMMARY ——————————

IFI16, a member of the IFN-inducible PYHIN-200 gene family, displays multifaceted activity due to its ability to bind to various target proteins and, in turn, modulate a variety cell functions including proliferation, differentiation, apoptosis/pyroptosis, senescence, and inflammation. The last few year have seen major advances in our knowledge of IFI16 antiviral activity and its role in the immune response. Indeed, a wealth of evidence now supports a key role of IFI16 in the activation of innate immunity and viral restriction against Herpesviruses and Lentiviruses, such that the definition of IFI16 as a 'restriction factor' is now widely accepted. However, most viruses have developed their own unique strategy to antagonize IFI16, leading to a modification or disruption of its function. This review summarizes our current understanding of how viral replication is sensed and then inhibited by IFI16 protein and the viral strategies employed to defeat this host defense mechanism. We will focus mainly on Herpesviruses, although recent discoveries on the role of IFI16 in lentiviral infection will also be considered.

KEY WORDS: IFI16, Intrinsic immunity, DNA sensors, Restriction factors, Antiviral defense, Viral escape mechanisms.

INTRODUCTION

The process of viral replication in the infected cell involves a complex interplay between viral and host factors. The innate immune response is the body's first line of defense against infection. Unlike the adaptive immune response, the innate response is not very specific and does not lead to long-lasting immunity. Classical innate immunity against viruses is mediated by specialized cells including natural killer cells, dendritic cells, and macrophages (Takeuchi and Akira, 2010; Iwasaki *et al.*, 2012). Recent studies have also revealed the existence of cellular-based defense mechanisms, now referred to as "intrinsic immunity", thus giving rise to a third branch of the traditionally bipartite immune system (Bieniasz *et al.*, 2004). Key components of the innate immune system comprise interferons (IFNs), in particular type I IFNs, and cytokines, secreted by most cell types following infection. The binding of interferons to their specific cell surface receptors induces the expression of a plethora of host factors that can interfere with various steps of the virus's replication cycle (Bieniasz *et al.*, 2004). Examples of these antiviral host factors include: the APOBEC3 family of cytidine deaminases (Mangeat *et al.*, 2003), Viperin (Chin and Cresswell, 2001), ISG15 (Skaug and Chen, 2010), TRIM5α (Grutter and Luban, 2012), SAMHD1 (Laguette and Benkirane, 2012), and bone marrow stromal antigen 2 (BST-2) (Hammonds *et al.*, 2012). The cellular proteins involved in antiviral activity are often called "restriction factors" (RFs) as they are germline-encoded factors that mediate a

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cell-intrinsic immune response against viral replication (Bieniasz *et al.,* 2003). Many of these RFs also behave as pattern recognition receptors (PRRs) since they recognize and directly bind to viral components (Baumann *et al.,* 2006; Paludan and Bowie, 2013; Orzalli and Knipe, 2014). However, unlike Toll-like receptors (TLRs) and RIG-like receptors (RLRs), which inhibit virus infection indirectly, RFs block viral replication by directly interfering with the activity of essential viral genes, often before activation of the IFN response.

Thus, RFs display several properties that differentiate them from pathogen sensors: RFs:

1) autonomously exhibit antiviral activity in culture-based assays and inhibit specific processes in viral replication;
2) are constitutively expressed in various cell types, but can be induced by IFNs;
3) are often antagonized by viral proteins (Malim and Bieniasz, 2012);
4) are subject to positive genetic selection driven by host-pathogen coevolution (Bozek and Lengauer, 2010; Compton *et al.,* 2012).

Thus, the concept of intrinsic immunity served as a basis to explain why defined cell lines are either 'restrictive' or 'permissive', depending on whether wild-type or mutant viruses efficiently replicate within them. The Retroviruses have provided a model system that has played a pivotal role in expanding our understanding of virus-host interactions (Takeuchi and Matano, 2008), but an increasing body of evidence suggests that similar mechanisms are also used by a number of other virus families including Herpesviruses (Lilley *et al.,* 2011; Paludan *et al.,* 2011; Tavalai and Stamminger, 2011; Boutell and Everett, 2013). On the other side of the coin, viruses themselves have also evolved mechanisms to inhibit and defend themselves against host RFs, and thus evade host restriction immunity (Roy and Mocarski, 2007; Duggal and Emerman, 2012; Yan *et al.,* 2012; Johnson *et al.,* 2013; Orzalli and Knipe, 2014).

Members of the PYHIN protein family are now recognized to play a central role in intrinsic immunity as sensors of microbial DNA and restriction factors against viral replication (Connolly and Bowie, 2014; Jakobsen and Paludan, 2014).

The present article will review the literature examining the role of the Interferon-inducible protein 16 (IFI16) in the control of viral replication, and the diverse strategies viruses have evolved to overcome IFI16 activity, ultimately leading to successful viral replication in target cells. The review will focus on Herpesviruses, the most extensively studied viruses in relation to IFI16 restriction activity, although recent findings related to HIV will also be discussed. The studies featured in this review provide compelling evidence supporting the prominent role of IFI16 in host antiviral defenses (Table 1).

TABLE 1 - *Overview of IFI16 role in antiviral response.*

Virus	*DNA sensing*	*Role in antiviral response*	*Viral evasion*	*References*
HSV-1	Cytoplasm	IFN type I production		Horan *et al.,* 2013 Unterholzner *et al.,* 2010
	Nucleus	IFN type I production	Degradation by HSV-1 ICP0	Cuchet-Lourenco *et al.,* 2013 Orzalli *et al.,* 2013, 2012
		Inflammasome activation		Johnson *et al.,* 2013
HCMV	Nucleus	Inhibition of HCMV-UL54 promoter	Delocalization upon phosphorylation by HCMV- UL97	Dell'Oste *et al.,* 2014 Gariano *et al.,* 2012
		Antiviral cytokine expression	Sequestration by HCMV-pp65	Li *et al.,* 2013
KSHV	Nucleus	Inflammasome activation		Singh *et al.,* 2013 Kerur *et al.,* 2011
EBV	Nucleus	Inflammasome activation		Ansari *et al.,* 2013
HIV-1	Cytoplasm	IFN type I production		Berg *et al.,* 2014
		Inflammasome activation		Monroe *et al.,* 2014 Jakobsen *et al.,* 2013

The PYHIN-200 family IFI16 protein

The IFN-inducible PYHIN-200 gene family encodes a class of homologous proteins that share a 200-amino acid signature motif (HIN) (Dawson and Trapani, 1995). Five human members have been identified (IFI16, MNDA, AIM2, PYHIN1, and PO3), while variable family expansion has been detected in other mammals, with at least thirteen family members present in mice (Brunette *et al.,* 2012; Cridland *et al.,* 2012; Connolly and Bowie, 2014; Jakobsen and Paludan, 2014). IFI16 displays two HIN domains (designated A and B) separated by a spacer region. The length of this region is regulated by alternative mRNA splicing, giving rise to three IFI16 isoforms (designated A-C) (Johnstone *et al.,* 1998). The HIN domains allow these proteins to bind both dsDNA and ssDNA in a sequence-independent manner (Dawson and Trapani, 1995; Yan *et al.,* 2008; Unterholzner *et al.,* 2010; Jin *et al.,* 2012). IFI16 also contains a homotypic protein-protein interaction PYRIN domain (PYD) at the N-terminus (Johnstone *et al.,* 1998; Liao *et al.,* 2011) (Figure 1).

The predominant B isoform of IFI16 is detectable in cells of different histological type, including human epithelial cells, fibroblasts, endothelial cells, and cells of hematopoietic origin (Johnstone *et al.,* 1998; Gariglio *et al.,* 2002, 2011; Wei *et al.,* 2003; Ouchi and Ouchi, 2008; Kis-Toth *et al.,* 2011). Accumulated experimental evidence has attributed multiple functions to IFI16 ranging from the DNA damage response, apoptosis and senescence promotion, cell growth, and cell differentiation regulation (reviewed in Gariglio *et al.,* 2011; Xin *et al.,* 2003; Zhang *et al.,* 2007; Gugliesi *et al.,* 2010; Song *et al.,* 2010; Duan *et al.,* 2011). IFI16 is also linked to the pathogenesis of certain autoimmune diseases, such as systemic lupus erythematosus (SLE), where it is secreted from cells as alarmin, and it forms a common autoantigen (Mondini *et al.,* 2006; Mondini *et al.*, 2007; Costa *et al.,* 2011; Caneparo *et al.,* 2013; Gugliesi *et al.,* 2013).

IFI16 was originally reported as a nuclear protein since it contains a nuclear localization signal (NLS) (Briggs *et al.,* 2001). However, in contrast to the exclusive nuclear or cytoplasmic localization pattern of the other 200-family proteins, the IFI16 protein has been detected in the nucleus (both in the nucleolus and in the nucleoplasm), the cytoplasm, and in both compartments in various cell types (Veeranki and Choubey, 2012). For example, upon exposure of human keratinocytes to UV-B irradiation, IFI16 is redistributed from the nucleus into the cytoplasm and into the cell culture supernatant (Costa *et al.,* 2011). Similar nucleus-to-cytoplasm translocation of IFI16 was also observed in skin sections obtained from patients with SLE, which may contribute to the detection of autoantibodies against IFI16 in the sera of these patients (Costa *et al.,* 2011).

Interestingly, IFI16-DNA binding activity leads to the activation of different signaling pathways and the subcellular localization of IFI16 is critical for determining which pathway is activated.
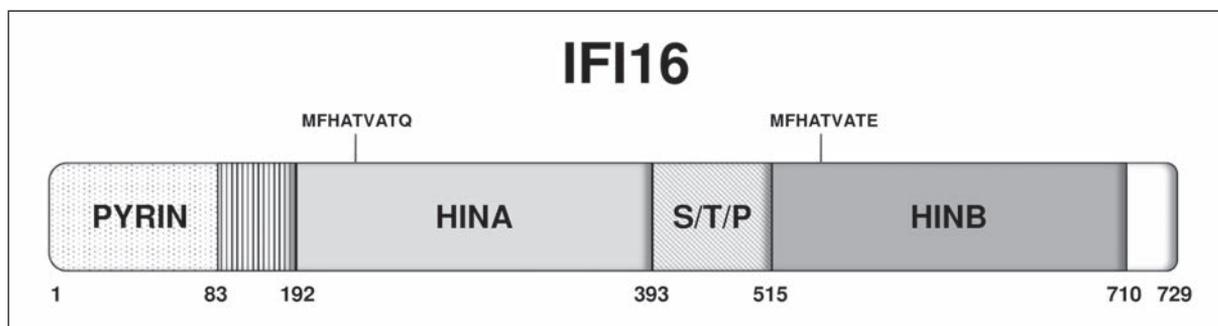

FIGURE 1 - *Schematic illustration of IFI16 protein structure. Residues 1-83 constitute the N-terminal PYRIN domain (dotted box). The solid-shaded boxes denote the HINA (residues 192-393) and the HINB (residues 515-710) domains. Diagonal stripped bars indicate the S/T/P-rich seven amino acid repeat motifs between the two HIN domains, resulting from alternative splicing of the gene. The conserved MFHATVAT motifs are also shown. Numbers located below each diagram represent amino acid positions.*

Protein-protein interactions have been recognized to influence the subcellular localization of proteins, and IFI16 protein can form homo- or heterodimers with several other proteins including: p53, Rb, BRCA1, ASC, and STING (Johnstone *et al.,* 2000; Aglipay *et al.,* 2003; Gugliesi *et al.,* 2005; Unterholzner *et al.,* 2010; Kerur *et al.,* 2011; Liao *et al.,* 2011). However, the molecular mechanisms regulating IFI16 redistribution between the nuclear and cytoplasmic compartments remain unknown.

The crystal structure of the IFI16 HIN-B domain was recently resolved in a complex with dsDNA, revealing non-sequence-specific DNA recognition, since binding occurs exclusively through electrostatic attraction between the positively charged HIN domain residues and the sugar phosphate backbone of double-stranded (ds) DNA (Jin *et al.,* 2012; Liu *et al.,* 2014). Finally, IFI16 was recently shown to bind cooperatively to dsDNA in a length-dependent manner and to cluster into distinct protein filaments, even in the presence of excess dsDNA (Morrone *et al.,* 2014). The isolated DNA-binding domains of IFI16 were found to form weak affinity associations with dsDNA without forming filaments, whereas the cooperative filament assembly only occurred in the presence of the PYRIN domain (Morrone *et al.,* 2014). This is consistent with the role of IFI16 as a viral DNA sensor and as a key mediator in driving the innate immune response.

IFI16 DURING VIRAL INFECTIONS

Alpha-Herpesviruses and IFI16
Herpes simplex virus type 1
Herpes simplex virus type 1 (HSV-1) is a common and highly contagious pathogen that results in productive infection in many cell types *in vitro* and establishes a latent infection *in vivo* (in the trigeminal ganglia of host organisms) from which it can be periodically reactivated resulting in recurrent lesions at the site of primary infection (Roizman *et al.,* 2006).
HSV-1 was the first member of the Herpesvirus family for which a role of IFI16 as an intracellular sensor of viral DNA was identified. During infection, the HSV-1 capsid is ubiquitinated in the cytosol and degraded by proteasomes, hence

releasing free HSV-1 genomic DNA into the host cell cytosol (Horan *et al.,* 2013). Although IFI16 remains predominantly nuclear, a significant quantity relocalizes to the cytosol and binds directly to isolated viral DNA motifs. STING, a mediator of IFN-β induction, is recruited to IFI16 that has sensed DNA and activates IRF-3, in turn driving the transcription of IFN- and IFN-stimulated genes (Unterholzner *et al.,* 2010; Horan *et al.,* 2013). However, it remains to be established whether IFI16 first recognizes HSV-1 DNA in the nucleus and then migrates to the cytoplasm to stimulate signal transduction of IFN-β and pro-inflammatory genes. Moreover, the specific features of dsDNA critical for IFI16 sensing and how dsDNA stimulates IFI16 to recruit STING are yet to be ascertained. The IFI16-mediated induction of pro-inflammatory gene transcription is reinforced by the observation that depletion of IFI16 by RNA interference inhibits DNA- and HSV-1-mediated gene induction as well as the activation of IRF3 and NF-κB (Unterholzner *et al.,* 2010). Moreover, IFN type I induction appears to be dependent on STING and other downstream components of this pathway in a variety of cell types, including human and mouse monocytic cell lines, mouse corneal epithelial cells (Conrady *et al.,* 2012), human primary, and immortalized fibroblasts (Duan *et al.,* 2011; Orzalli *et al.,* 2012), human primary macrophages (Soby *et al.,* 2012; Horan *et al.,* 2013), neutrophils (Tamassia *et al.,* 2012), and dendritic cells (Kis-Toth *et al.,* 2011).
By contrast, other studies have found that upon sensing HSV-1 DNA, IFI16 remains in the nucleus and activates different pathways to counteract HSV-1 replication. In HSV-1 infected primary human foreskin fibroblasts (HFF), IFI16 triggers the STING-mediated activation of IRF3, leading to IFN-β production. As IFI16 remains in the nucleus during this process, the authors hypothesized the existence of an unknown downstream factor shuttling from nuclear IFI16 to cytoplasmic STING (Orzalli *et al.,* 2012).
Finally, a role of IFI16 has been identified in the recruitment of known HSV-1 restriction factors, such as PML nuclear body (PML NB) components, to foci closely associated with viral genomes (Everett and Murray, 2005). This recruitment action of IFI16 further strengthens

its restrictive effects on HSV-1 infection, although it occurs via a very distinct mechanism from that of IRF3-mediated interferon induction, and which may be linked to the PML NB response to viral infection (Cuchet-Lourenco *et al.,* 2013).

To counteract IFI16 antiviral activity, HSV-1 specifically targets IFI16 for rapid proteasomic degradation, using a mechanism dependent on the HSV-1 E3 ubiquitin ligase ICP0, an immediate early protein of HSV-1 (Orzalli *et al.,* 2012; Orzalli *et al.,* 2013; Johnson *et al.,* 2013). When encapsidated viral DNA generated by HSV d106 mutant, expressing very high levels of ICP0 but no other IE proteins, is released into the nucleus and sensed by nuclear IFI16, activation of the IRF3 pathway is inhibited. Conversely, upon infection with HSV d109 (mutated in the immediate-early genes: ICP0, ICP4, ICP22, ICP27, and ICP47), induction of the IRF-3-responsive genes is maintained, confirming that ICP0 is the viral component responsible for the escape from IFI16-mediated IFN induction (Orzalli *et al.,* 2012). In agreement with these data, IFI16 has been demonstrated to act as a restriction factor against ICP0-null HSV-1, limiting viral replication and immediate-early gene expression (Orzalli *et al.,* 2012). The ability of IFI16 to silence HSV-1 IE genes relies on the conformational change of the protein that follows its binding to nucleosome-free DNA in the nucleus, and leads to the release of the PYRIN domain from an autoinhibited state (Jin *et al., 2012*). This allows activated IFI16 to signal from the nucleus to the cytoplasm, triggering innate immune signaling pathways and the recruitment of histone modification enzymes that promote heterochromatic rearrangements on the exogenous DNA and chromatin compaction, finally resulting in the silencing of viral IE genes (Orzalli *et al.,* 2013).

The factors governing the degradation of IFI16 and its colocalization with ICP0 are distinct from those of PML, a well-characterized ICP0 substrate (Everett and Murray, 2005). Unlike PML, the colocalization of IFI16 with ICP0 is dependent on the RING finger mediated E3 ubiquitin ligase activity of ICP0, and colocalization does not occur when proteasome activity is inhibited. ICP0 expression in the absence of infection does not destabilize IFI16, and

degradation occurs efficiently in the absence of ICP0 if infection is progressing efficiently. Thus, ICP0 by itself is not sufficient for HSV-1-induced degradation of IFI16; instead, other cellular factors are likely to be involved.

More recently, Li *et al.* (2012) demonstrated that the acetylation status of IFI16 represents a critical determinant of the ability of the protein to sense HSV-1 DNA in the nuclei of U2OS epithelial cells. The acetylation status of IFI16 was shown to be a major determinant of IFI16 subcellular distribution, and acetylation within the nuclear localization sequence (NLS) of IFI16 was found to promote cytoplasmic localization and inhibit its nuclear import. In addition, inhibition of deacetylase activity by deacetylase inhibitors prevented the nuclear accumulation of IFI16, implicating a role of histone deacetylases (HDACs) in the regulation of IFI16 localization. Despite the major advances made over recent years in the field of HSV-1 immune sensing, many questions remain regarding the role of IFI16 in innate antiviral defense mechanisms against Herpesviruses *in vivo*, including its role in directing adaptive immune responses (Figure 1, upper left quadrant).

Beta-Herpesviruses and IFI16

Human Cytomegalovirus

During infection by Human Cytomegalovirus (HCMV), a prototype member of the β-subgroup of Herpesviruses (Tandon and Mocarski, 2012; Griffiths *et al.,* 2014), IFI16 acts as both a nuclear DNA sensor and a restriction factor and plays a critical role in triggering the antiviral response.

IFI16 obtained from extracts of uninfected HeLa cells was first demonstrated to bind in a non-sequence-specific manner to the UL54 promoter of HCMV (Luu and Flores, 1997); IFI16 was then demonstrated to inhibit UL54 activity in a reporter assay performed in uninfected cells (Johnstone *et al.,* 1998). Since the experiments were conducted in uninfected cells, no information could be inferred on the antiviral state induced by IFI16. This view of IFI16 has changed over recent years thanks to the findings generated by our group, demonstrating how IFI16 acts as a restriction factor for HCMV replication (Gariano *et al.,* 2012). We have shown that silencing IFI16 expression in hu-

man embryo lung fibroblasts (HELFs) through the use of small interfering RNA, or inactivating IFI16 protein by introducing a Lentivirus expressing a dominant negative mutant form

lead to significantly enhanced HCMV replication. Conversely, IFI16 overexpression caused a strong decrease in viral production. Interestingly, transfection experiments using the luciferase
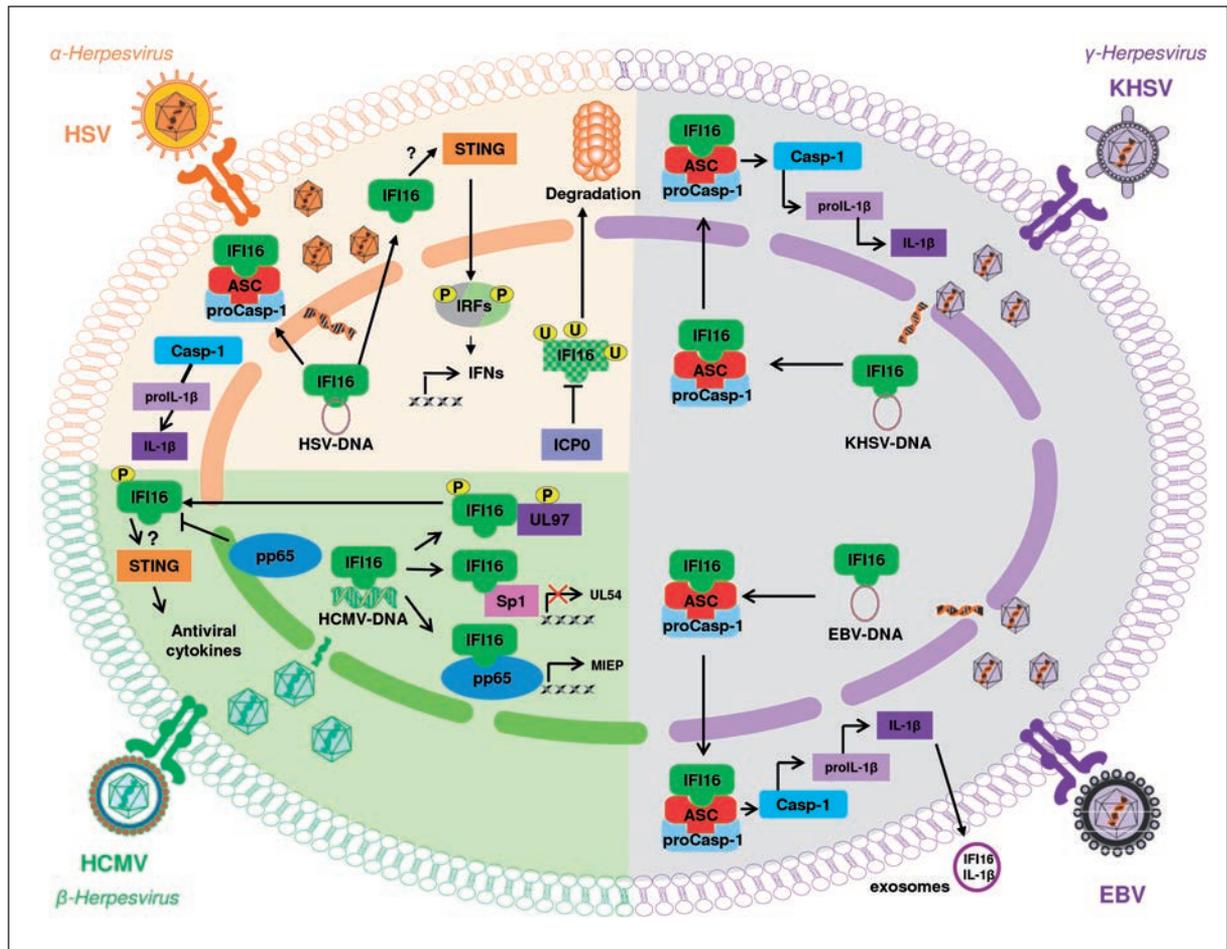

FIGURE 2 - *Critical steps for IFI16-mediated control of Herpesvirus infections. Upper left quadrant. The HSV-1 genome is released into the nucleus, it circularizes and is recognized by IFI16. IFI16 exits the nucleus and assembles with the adaptor protein ASC and procaspase-1 (proCasp-1) to form a functional inflammasome complex, which causes the cleavage and activation of caspase-1 (Casp-1) and IL-1β. IFI16 delocalization from the nucleus also stimulates the nuclear-to-cytoplasmic signaling cascade, activating IRF-3, which dimerizes and translocates to the nucleus to activate IFN type I expression. HSV-1 immediate-early protein ICP0 promotes proteasome degradation of IFI16. Bottom left quadrant. IFI16 detects HCMV-DNA in the nucleus. In the early phases of infection, HCMV-pp65 hijacks IFI16 to activate MIEP expression; later-on IFI16 inhibits HCMV replication by blocking the activity of Sp1-like transcription factors on the viral UL54 promoter. To evade IFI16 antiviral activity, HCMV induces nuclear egression of IFI16 upon binding of viral protein kinase UL97 to IFI16. In the cytoplasm, IFI16 activates STING-mediated antiviral cytokine expression. Right quadrant. Upon sensing the KHSV nuclear DNA, IFI16 protein recruits ASC and proCasp-1 to form a functional inflammasome in the nucleus, which subsequently exits the nucleus to cause pro-IL-1β processing in the cytoplasm. During primary infection of B or epithelial cells, the EBV released nucleocapsid travels to the nuclear pore, and linear DNA enters the nucleus, circularizes, and is sensed in the infected cell during latency by IFI16, leading to recruitment of ASC and proCasp-1 to form an inflammasome complex; this is followed by its translocation to the cytoplasm, activation of Casp-1, and cleavage of pro-IL-1β into its mature forms. IFI16 and cleaved IL-1β are sorted and released from cells via exosomes, which may constitute an EBV-mediated strategy to subvert their inflammatory functions.*

reporter gene driven by deleted or site-specific mutated forms of the HCMV DNA polymerase (UL54) or the DNA polymerase processivity factor UL44 promoters demonstrated the inverted repeat element 1 (IR-1) to be the target DNA motif of IFI16 suppression. The transcription factor Sp1 was identified as the partner of IFI16 in the suppression of the UL54 promoter. Together, these results demonstrate that in addition to the previously recognized role of IFI16 as a DNA sensor, it also acts as a restriction factor of Herpesvirus replication (Gariano *et al.*, 2012).

Suppression of UL54 promoter transactivation by IFI16 is apparently at variance with the findings of Cristea *et al.* (Cristea *et al.*, 2010), who showed that pp65 interacts with and recruits IFI16 to the major immediate-early promoter (MIEP), stimulating, rather than inhibiting, MIEP activity. Moreover, they found that infection of IFI16 knockdown cells with HCMV resulted in decreased levels of immediate-early transcripts compared with control cells and resulted in progeny with delayed growth kinetics (Cristea *et al.*, 2010). This discrepancy can be explained considering recent results obtained by our group, directed at understanding how HCMV is able to antagonize IFI16 restriction activity (Dell'Oste *et al.*, 2014). Early on during infection, IFI16 binds to viral DNA. However, at a later time point during viral DNA synthesis, IFI16 is mislocalized from the nucleus to the cytoplasm. The molecular mechanisms underlying this virus-induced nuclear egression of IFI16 require the binding of viral protein kinase UL97 to IFI16. Upon binding, IFI16 undergoes phosphorylation, which in turn promotes its nucleo-cytoplasmic relocalization. Thereafter, the ESCRT (Endosomal Sorting Complex Required for Transport) machinery regulates the translocation of IFI16 into the virus assembly complex (AC). Finally, during the virus maturation step, a percentage of IFI16 becomes incorporated into the new virions budding from the cells. Thus, to evade IFI16 restriction activity, HCMV has evolved a strategy that involves the nuclear egression of IFI16 followed by its sequestration into newly formed virions (Dell'Oste *et al.*, 2014). This may well be the event leading to inactivation of IFI16 antiviral activity. Another viewpoint to consider is that the IFI16 incorporated into mature virions may actually promote viral replication during early infection, for instance by activating the MIEP promoter as suggested by Cristea *et al.* (Cristea *et al.*, 2010) but then the virus traps it within mature virions to avoid its restriction activity.

Concerning the ability of IFI16 to sense HCMV-DNA, Li *et al.* (Li *et al.*, 2013) have provided strong evidence supporting the role of IFI16 as a nuclear DNA sensor during early HCMV infection. They show that IFI16 binds viral DNA and triggers the STING/Tank-binding kinase (TBK1)/IRF3 signaling pathway to induce the release of antiviral cytokines. Again, the pp65-IFI16 interaction is crucial for modulating the outcome of HCMV infection. pp65 sequesters the IFI16 PYRIN domain via the concerted action of its N and C termini, blocking nuclear IFI16 oligomerization and subsequent immune signaling pp65 has similar inhibitory effects on all other nuclear PYHIN proteins (i.e., PYHIN1 and MNDA). Additionally, site-specific phosphorylation by host kinases compromises pp65-mediated PYRIN interference.

A distinct scenario is observed in HSV-1 infection, which uses the viral E3 ubiquitin ligase ICP0 to help target IFI16 for degradation, thereby inhibiting type I IFN (Orzalli *et al.*, 2012) and the inflammatory response (Johnson *et al.*, 2013). By contrast, HCMV adopts a non-destructive strategy that may support its longer lytic cycle better. As no homologs of pp65 are present in other Herpesvirus subfamilies, the pp65-dependent immune evasion is likely to be specific to HCMV. Overall, the distinct viral mechanisms used to target IFI16 underscore the essential role of this nuclear DNA sensor in the innate antiviral response (Figure 2, bottom left quadrant).

Gamma-Herpesviruses and IFI16
Kaposi's Sarcoma-Associated Herpesvirus
Kaposi´s sarcoma-associated Herpesvirus (HHV-8; KSHV) is a lymphotropic virus that establishes an asymptomatic latent infection, except in immunocompromised patients where it can lead to Kaposi´s sarcoma (KS), primary effusion lymphoma (PEL), and some forms of multicentric Castleman´s disease (MCDs) (Ganem *et al.,* 2006). Like all Herpesviruses, KSHV binds to and fuses with the cell membrane, re-

leasing its DNA-containing capsid into the cytosol, where it is then rapidly delivered into the nucleus. Viral DNA enters the nucleus, where it replicates (in the case of lytic infection) or remains dormant (establishing a latent infection), maintaining an episomal circular DNA genome in the nucleus of infected cells (Chandran *et al.,* 2010). KSHV was the second DNA virus, after HSV-1, for which the function of IFI16 as innate immune DNA sensor was demonstrated (Kerur *et al.,* 2011). In contrast to the HSV-1 model (Unterholzner *et al.,* 2010), Kerur *et al.* (2011) s study identified IFI16 protein as a nuclear sensor for viral DNA in endothelial cells, further reinforcing the evidence indicating that IFI16 protein, depending upon the viral and cell type, is able to sense viral DNA in the cytoplasm and the nucleus, thereby initiating innate immune responses. Upon sensing the viral DNA in the nucleus, IFI16 protein recruits the adaptor molecule ASC and procaspase-1 via their respective PYD domains to form a functional inflammasome that subsequently exits from the nucleus into the cytosol to cause pro-IL-1β processing. The role of IFI16 in the transcriptional regulation of pro-IL-1β and IL-6 transcription was confirmed by the observation that IFI16 but not AIM2 silencing in endothelial cells resulted in decreased caspase-1 activation and compromised expression of IL-1β and IL-6. Both pro-IL-1β and IL-6 are NF-κB-dependent genes, consistent with the previously described function of IFI16 as an activator of this transcription factor during HSV-1 infection (Unterholzner *et al.,* 2010).

KSHV establishes a lifelong latent infection characterized by the persistence of multiple viral episomal genomes with minimal gene expression in the host cell nuclei, during which it must evade immune detection. Emerging lines of evidence indicate that the innate immune response plays a central role in regulating the latency program of gamma Herpesviruses, including KSHV, but it remains to be established whether such latency-associated nuclear viral genomes activate an innate immune response. The role of IFI16 in KSHV latent infections has recently been investigated (Singh *et al.,* 2013) using endothelial telomerase-immortalized human umbilical cells (TIVE) supporting KSHV stable latency (TIVE-LTC cells) and PEL (cavi-ty-based B-cell lymphoma 1 [BCBL-1]) cells. In these cells, the KSHV genome colocalizes with nuclear IFI16, triggering an intrinsic immune response. The sensing of the viral genome by IFI16 culminates in the formation of an inflammasome complex with ASC and caspase-1 and the subsequent cleavage and maturation of pro-IL-1β and pro-IL-18. These findings suggest the existence of a constant IFI16-mediated innate defense response against the KSHV genome during latency, which could be one of the potential mechanisms for the inflammatory and angiogenic responses detected in KSHV-associated malignancies. Moreover, IFI16 and cleaved (but functionally inactive) IL-1β were detected in the exosomes released from BCBL-1 cells. This could represent a potential mechanism by which KSHV subverts the host innate response by inhibiting the constitutive secretion of pro-apoptotic mature IL-1β, which may otherwise challenge the establishment of viral latency. Finally, Singh and colleagues (Singh *et al.,* 2013) also detected IFI16 colocalization with ASC in an *in vivo* system, consisting of KSHV-infected solid PEL lesions, suggesting the potential involvement of an IFI16-mediated inflammasome in KSHV pathogenesis *in vivo*. These findings candidate IFI16 as a potential pharmaceutical target for decreasing inflammation associated with KSHV as well as for eradicating KSHV latent infection and its associated diseases (Figure 2, right quadrant).

Epstein-Barr Virus

Epstein-Barr Virus (EBV; HHV-4), a gamma-1 human Herpesvirus, is an opportunistic human pathogen that infects more than 95% of individuals worldwide by adulthood. EBV is etiologically associated with the benign lymphoproliferative disease infectious mononucleosis, as well as with the development of numerous B lymphocyte and epithelial cell malignancies, such as Burkitt, Hodgkin, AIDS-associated, post-transplant lymphomas, and nasopharyngeal carcinoma (Maeda *et al.,* 2009). EBV possesses a biphasic life cycle, comprising a lytic and a latent phase, which facilitates its episomal genome persistence and its evasion of host immune responses. However, in contrast to HSV-1, its latent state is characterized by the expression of a set of proteins necessary for

the establishment and maintenance of latency (Amon and Farrell, 2005). The ability of EBV to establish three different types of latency has been known for many years, but it remains to be established whether the persistent EBV genome is recognized by the host innate sensing mechanism and, if so, whether it induces an innate immune response. These mechanisms were elucidated by experiments carried out by Ansari *et al.* (2013), who demonstrated inflammasome activation in cellular models representative of the three types of EBV latency: latency I Raji cells, latency II NPC C666-1 cells, and latency III lymphoblastoid cell lines (LCL). In these models, IFI16 behaves as a nuclear DNA sensor for the EBV genome, colocalizing with the viral DNA in infected cell nuclei. This leads to the formation of the inflammasome complex IFI16-ASC-procaspase-1 in all three cell models of EBV latency, followed by its translocation to the cytoplasm, where caspase-1 activation brings about the release of inflammatory cytokines IL-1β, IL-18, and IL-33 by cleavage of their proproteins. Although EBV nuclear antigen 1 (EBNA1) and EBV-encoded small RNAs (EBERs) are common to all forms of EBV latency, caspase-1 cleavage was not detected in cells expressing EBNA1 alone. Moreover, blocking EBER transcription did not inhibit caspase-1 cleavage. Finally, IFI16 and cleaved caspase-1, IL-1β, IL-18, and IL-33 were shown to be sorted to the exosomes from Raji and LCL cells, which could constitute the strategy employed by EBV to evade the inflammatory functions of these cytokines. Interestingly, in contrast to HSV-1 and HCMV infections, IFI16 levels were not altered in cells latently infected with EBV and KSHV. This finding suggests that although the IFI16 inflammasome results from the host's innate response to infection, EBV and KSHV might have evolved to utilize IFI16 in certain cell types for their survival advantage during latency. This possibility provides new future lines of enquiry in our attempt to understand the dynamic links between host innate responses and viral latency (Figure 2, right quadrant).

HIV and IFI16

The early innate sensing of HIV infection and activation of antiviral responses, including type I IFN production, play a central role in early antiviral defense against HIV in the pathogenesis of AIDS, restricting viral replication and spread (Gougeon Herbeuval, 2012; Iwasaki *et al.*, 2012). On the other hand, it is well established that the secretion of cytokines and IFN during innate immune responses contributes to the chronic activation of the immune response, the development of immunodeficiency, and the progression toward AIDS (De Cock *et al.*, 2012). HIV-1 and other lentiviruses replicate through a life cycle involving steps in which the genomic information is carried in the form of single-stranded (ss) RNA, an RNA-DNA hybrid, ssDNA, and dsDNA (Abbink and Berkhout, 2008). Several PRRs sensing HIV-1 RNA have been reported, including TLR-7 and Retinoic-inducible gene-I (RIG-I), also in cell types not normally permissive to HIV-1 infection, indicating that nucleic acids are potent stimulators of innate immune responses (Heil *et al.*, 2004; Solis *et al.*, 2011; Berg *et al.*, 2014; Van Montfoort *et al.*, 2014). Cellular restriction factors, most of which are IFN-stimulated genes controlling HIV replication at different stages of the replication cycle, have been characterized, such as APOBEC, tetherin, the Schlafen protein SLFN11, and the recently reported histidine-aspartic (HD) domain-containing protein 1 (SAMHD1) (Strebel *et al.*, 2009; Gao *et al.*, 2013).

Very recent studies successfully investigated the role of IFI16 in HIV infection, using cellular models consisting of primary human macrophages or CD4+ T cells (Jakobsen *et al.*, 2013; Berg *et al.*, 2014; Monroe *et al.*, 2014). In primary human macrophages, synthetic DNA oligonucleotides corresponding to DNA forms of the lentivirus replication cycle and ssDNAs derived from HIV-1 proviral DNA potently induce the expression of IFNs. This response was stimulated by stem regions in the DNA structure and was dependent on IFI16, which directly bound to immunostimulatory DNA with high affinity, but not to a less stimulatory, modified form of ssDNA. This led to the activation of the STING-TBK1-IRF3/7 pathway. Moreover, IFI16 colocalized and associated with lentiviral DNA in the cytoplasm of macrophages. In contrast, replication of HIV-1 was elevated in cells with decreased expression of both IFI16 and STING. Altogether, these findings suggest that IFI16

acts as a sensor for lentivirus DNA forms produced during the lentiviral replication cycle, and that it contributes to the early control of HIV-1 replication in macrophages, preventing virus-induced cytopathic effects. Because macrophages are the first cell type infected during HIV-1 infection, these data suggest that IFI16 may be the first PRR sensor to encounter the virus upon entry.

While macrophages represent an important viral reservoir, activated CD4+ T cells constitute the most permissive cell types supporting high levels of viral replication. Many findings have been reported on the function of T cells and adaptive immunity during HIV infection, while the interactions between HIV and the innate immune system have only recently been recognized to play a central part in the pathogenesis of HIV infection (Mogensen *et al.,* 2010). The role of IFI16 in innate immunity against HIV in CD4+ T cells is the topic of recent investigations performed by two independent groups (Berg *et al.,* 2014; Monroe *et al.,* 2014). In contrast to the responses of primary macrophages and THP-1 cells (Jakobsen *et al.,* 2013), human primary T cells fail to induce strong IFN reac-
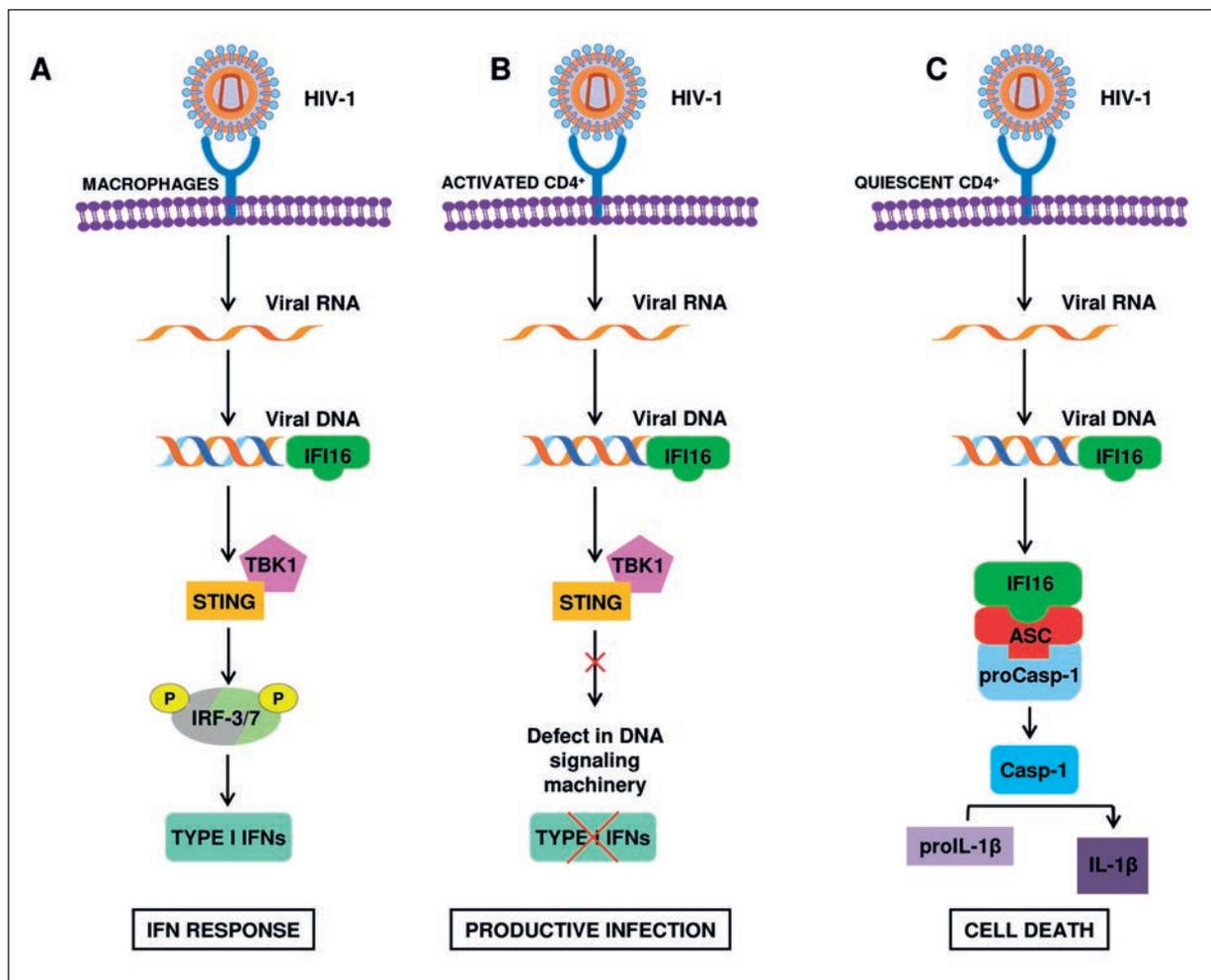

FIGURE 3 - *Working models of IFI16 DNA sensing during HIV-1 infection. (A) In primary human macrophages, IFI16 binds directly to dsDNA derived from HIV-1 infection. The STING-TBK1-IRF3/7 pathway is activated, leading to type I IFN production. (B) In activated CD4+ cells, IFI16 senses HIV-1 DNA in the cytoplasm, where it recruits STING and TBK1 (but without impacting IFN expression), resulting in HIV-1 productive infection. (C) In quiescent CD4+ cells, IFI16 detects HIV-1 dsDNA, with consequent inflammasome activation. This results in the secretion of the highly inflammatory cell signaling molecule IL-1β and cell death by pyroptosis.*

tions affecting HIV replication upon the introduction of synthetic DNA into the cytoplasm, despite the fact that this cell type expresses key molecules involved in DNA signaling pathways. Instead, upon sensing foreign DNA in the T cell cytoplasm, IFI16 recruits STING and TBK1, but this does not affect the expression of IFN, IFN-stimulated genes, or pro-inflammatory cytokines. IFN responses, however, are evoked through the RIG-I pathway. Altogether, these findings suggest that the DNA signaling machinery is partly defective in T cells, resulting in an impaired ability to respond to HIV-DNA and to stimulate antiviral IFN responses, in turn contributing to the high permissiveness of CD4+ T cells to HIV-1 replication (Berg *et al.,* 2014).

Another strategy employed by IFI16 to modulate HIV replication relies on its ability to trigger CD4+ T cell death upon HIV infection, thus blocking virus replication. This strategy was elucidated thanks to the use of proteomic and targeted biochemical approaches, as well as two independent methods of lentiviral short hairpin RNA-mediated gene knockdown in primary CD4+ T cells (Monroe *et al.,* 2014). When the authors inhibited IFI16, CD4+ T cell death was avoided to an extent similar to that achieved using drugs that block HIV-1 DNA production. The IFI16-depleted CD4+ T cells also displayed lower amounts of active caspase-1 and inflammatory cytokines, consistent with decreased pyroptosis, demonstrating the major role of IFI16 in HIV infection (Doitsh *et al.,* 2014; Monroe *et al.,* 2014).

Taken together, these studies provide strong mechanistic insight into how HIV interacts with the DNA sensing system *in vitro*. Using PBMCs from HIV positive individuals, irrespective of whether patients were receiving antiretroviral treatment, an impaired expression of interferon-stimulated genes was observed in response to DNA (Nissen *et al.,* 2014). Interestingly, the expression level of IFI16 was increased in treatment-naïve patients. This was correlated with high viral load, low CD4 cell count, and CD38 expression in CD4+ central memory and effector memory T cells, a marker of immune activation. These data not only confirm the previous results obtained *in vitro* (Monroe *et al.,* 2014), but also reinforce the role of IFI16-mediated

DNA sensing and signaling to drive chronic immune activation, rather than being a general marker only, with possible implications for the control of opportunistic infections, chronic immune activation, and T cell death (Nissen *et al.,* 2014).

Together, these results highlight the role of IFI16 in the context of HIV infection, although a completely different scenario emerges when compared with that of Herpesviruses. IFI16 restricts viral replication in the case of Herpesviruses, and the finding that IFI16 is targeted for degradation by Herpesviruses further highlights the evolutionary pressure to counteract its activity. On the other hand, in the case of HIV infection, IFI16 triggers an innate immune response that, rather than protecting the host, drives the debilitating depletion of CD4+ T cells, followed by progression to AIDS (Figure 3).

CONCLUSIONS

To date, at least ten proteins have been reported to sense viral DNA or RNA and to behave as restriction factors, the signaling events and functions of which are distinctly regulated at several levels (Bieniasz *et al.,* 2003; Thompson *et al.,* 2011; Nie, Wang 2013). Of the DNA sensors, IFI16 is unique in that it shuttles between the nucleus and the cytoplasm, and it appears to be able to sense DNA in both compartments, depending on the localization of its DNA ligands (Cristea *et al.,* 2010; Unterholzner *et al.,* 2010; Kerur *et al.,* 2011; Li *et al.,* 2012; Horan *et al.,* 2013; Orzalli *et al.,* 2012, 2013; Johnson *et al.,* 2013; Singh *et al.,* 2013). The ability of IFI16 to detect DNA viruses such as HSV-1 and HCMV in the nucleus challenges the long-held assumption that "foreign" DNA is sensed simply by virtue of its presence in the cytosol. Given the proposed ability of IFI16 to act as a DNA sensor both in the cytosol and the nucleus, it is of utmost importance to increase our understanding of what factors control the subcellular localization of IFI16. Significant research efforts have been made in this direction. For instance, acetylation of the IFI16 nuclear localization signal has been identified to promote the cytoplasmic localization of IFI16 (Li *et al.,* 2012). Indeed, it will be interesting to learn

more about how cells and microbes control this and other post-translational modifications of IFI16.

Over the last decade, much progress has been made in unraveling the mechanisms of intracellular DNA-mediated innate responses. The discovery of IFI16 as a key modulator of both DNA- and RNA-triggered innate immune signaling will undoubtedly facilitate our understanding of these innate signaling processes. Given these observations, it will be of interest to evaluate the importance of IFI16 in stimulating host defenses against other types of DNA pathogens including parasites such as malaria. Another critical aspect will be to determine the extent to which IFI16/p204 is involved *in vivo* in the sensing of DNA during infection with viruses or intracellular bacteria, but this awaits the generation of mice lacking the p204 receptor. Other important issues include clarification of the key mediators of the IFI16-related pathway. Another major challenge will be to determine the role of IFI16-dependent signaling in specific diseases related to microbial infections, such as autoimmune disorders, and to ascertain whether such pathways can be targeted as a treatment strategy. Indeed, it will be critical to verify whether stimulation of these pathways is able to facilitate vaccination or anti-pathogen prophylactic strategies.

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