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International Journal of Biochemistry and Cell Biology 122 (2020) 105738

Contents lists available at ScienceDirect

International Journal of Biochemistry and Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Review article

Viroporins and inflammasomes: A key to understand virus-induced inflammation

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ARTICLE INFO

Keywords:
Viroporins
Inflammasomes
NLRP3
Inflammation
Virus-immunity

ABSTRACT

Viroporins are virus encoded proteins that alter membrane permeability and can trigger subsequent cellular signals. Oligomerization of viroporin subunits results in formation of a hydrophilic pore which facilitates ion transport across host cell membranes. These viral channel proteins may be involved in different stages of the virus infection cycle. Inflammasomes are large multimolecular complexes best recognized for their ability to control activation of caspase-1, which in turn regulates the maturation of interleukin-1 β (IL-1β) and interleukin 18 (IL-18). IL-1β was originally identified as a pro-inflammatory cytokine able to induce both local and systemic inflammation and a febrile reaction in response to infection or injury. Excessive production of IL-1β is associated with autoimmune and inflammatory diseases. Microbial derivatives, bacterial pore-forming toxins, extracellular ATP and other pathogen-associated molecular patterns trigger activation of NLRP3 inflammasomes. Recent studies have reported that viroporin activity is capable of inducing inflammasome activity and production of IL-1β, where NLRP3 is shown to be regulated by fluxes of K^+, H^+ and Ca^{2+} in addition to reactive oxygen species, autophagy and endoplasmic reticulum stress. The aim of this review is to present an overview of the key findings on viroporin activity with special emphasis on their role in virus immunity and as possible activators of inflammasomes.

1. Innate immune response to viral infections

The immune response to different pathological invasions in the human body is mediated by two arms; innate and acquired immunity. Innate immunity is the first arm of immune response to viral infections. The antiviral response is triggered upon the sensing of the different viral antigens by pattern recognition receptors (PRRs) of the infected cell (Bowie and Unterholzner, 2008). Such response is crucial as it acts to suppress viral replication and spread to other cells. It is also required for programming functional adaptive immune responses and, therefore, coordinates the entire host immune response to infection (Bowie and Unterholzner, 2008). The innate immune response to viruses is

Abbreviations: AIM, Absent in melanoma; ASC, Apoptosis-associated speck-like protein containing a carboxy-terminal CARD; ATP, Adenosine triphosphate; BMDC, Bone marrow derived dendritic cells; BMM, Bone marrow derived macrophages; CARD, Caspase activation and recruitment domain; CD, Cluster of differentiation; CoV, Coronavirus; CSFV, Classical swine fever virus; DAMPs, Danger associated molecular patterns; DC, Dendritic cells; EMCV, Encephalomyocarditis virus; HCV, Hepatitis C virus; HIV, Human immune deficiency virus; HMA, 5-(*N,N*-Hexamethylene)amiloride; IAV, Influenza A virus; IFNc, Interferon c; IL-18, Interleukin 18; IL-1R, Interleukin-1 beta receptor; IL-1RA, Interleukin 1 receptor antagonist; IL-1β, Interleukin-1 beta; IPAF, Ice protease-activating factor, also known as NLRC4; IRF3, Interferon regulatory transcription factor; LGP2, laboratory of genetics and physiology 2; LPS, Lipopolysaccharide; LRR, Leucine rich repeats; MAL, MyD88- adaptor-like; MAM, Mitochondrial-associated membrane; MAVS, Mitochondrial antiviral signaling protein; MDA5, Melanoma differentiation-associated protein 5; MERS, Middle East respiratory syndrome-related coronavirus; MHC, Major histocompatibility complex; MSU, Monosodium urate; MyD88, Myeloid differentiation primary response 88; NADPH, Nicotinamide adenine dinucleotide phosphate; NALP, NACHT, LRR and PYD domains-containing protein; NFκB, Nuclear factor kappa b; NLR, Nucleotide-binding domain, leucine-rich repeat; NLRP3, NLR family, pyrin domain containing 3; Nod, Nucleotide oligomerization domain; PAMP, Pathogen associated molecular patterns; PRR, Pattern recognition receptors; PYD, Pyrin domain; RIG-1, Retinoic acid-inducible gene I; RLR, RIG-1 like receptor; ROS, Reactive Oxygen species; RSV, Respiratory syncytial virus; SARS, Severe acute respiratory syndrome; TGN, Trans-golgi network; Th1, T helper 1 cells; Th17, T helper 17 cells; TICAM1, TIR-domain-containing molecule 1; TIR, Toll-interleukin 1 receptor; TIRAP, TIR-associated protein; TLR, Toll like receptor; TNF, Tumor necrosis factor; TRAM, TRIF-related adaptor molecule; TRIM25, Tripartite motif-containing protein 25; TRIF, TIR-domain-containing adaptor protein- inducing IFN-b

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https://doi.org/10.1016/j.biocel.2020.105738
Received 2 December 2019; Received in revised form 5 March 2020; Accepted 6 March 2020
Available online 07 March 2020
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composed of three main classes of PRRs, termed the RIG-I (retinoic acid-inducible gene I)-like receptors (RLRs), the toll-like receptors (TLRs), and the Nod (nucleotide oligomerization domain)-like receptors (NLRs) (Wilkins and Gale, 2010). These receptors sense specific molecules within the virus termed PAMPs (Pathogen associated molecular patterns) and then signal through various downstream messengers to activate transcription factors that drive the expression of antiviral genes and various cytokines, such as type I and III interferons and IL-1β (Horner, 2014). Production of these cytokines is essential for activation of the whole immune response against the viral infection. T ype I interferons induce expression of co-stimulatory molecules such as CD80, CD86 and CD40 leading to maturation of dendritic cells (DCs) as well as antigen presentation on MHC class I and cross presentation of viral antigens (Gessani et al., 2014). These cytokines are also responsible for recruitment of monocytes and other lymphocytes to the site of infection, and they upregulate effector molecules in charge of protein synthesis, cell growth and survival and activation of anti-viral mode (Arango Duque and Descoteaux, 2014).

1.1. Recognition of viruses by TLRs

Toll like receptors (TLRs) are expressed on various immune cells, including macrophages, dendritic cells (DCs), B cells, specific types of T cells, and even on non-immune cells such as fibroblasts and epithelial cells. To date, thirteen members of TLR family have been identified. TLRs belong among type I integral membrane glycoproteins, which comprise an extracellular domain containing different numbers of leucine-rich-repeat (LRR) motifs, a single transmembrane helix, and a cytoplasmic signaling domain homologous to that of the interleukin 1 receptor (IL-1R), called the Toll-interleukin 1 receptor (TIR) domain (Chang et al., 2007). Based on their primary sequences, TLRs can be divided into several subfamilies, each of which recognizes related PAMPs: the subfamily of TLR1, TLR2, and TLR6 recognizes lipids, whereas the closely related TLR7, TLR8, and TLR9 recognize nucleic acids (Celhar and Magalhaes, 2012). TLR 2, 3, 4, 7, 8 and 9 have been shown to be involved in virus recognition depending on the type of the genetic material which the virus possesses: single stranded RNA (ssRNA) is recognized by TLR7 and TLR8, double stranded (ds) RNA is recognized by TLR3 and viral DNA is recognized by TLR9 (Akira et al., 2006). After binding their ligands, TLRs dimerize and undergo conformational changes required for the recruitment of TIR-domain-containing adaptor molecules. Four downstream adaptor molecules are involved, resulting in differential response which are (1) Myeloid differentiation primary response 88 (MyD88), (2) Toll-interleukin 1 receptor (TIR) -associated protein (TIRAP)/MyD88- adaptor-like (MAL), (3) TIR-domain-containing adaptor protein- inducing IFN-β (TRIF) /TIR-domain-containing molecule 1 (TICAM1) and (4) TRIF-related adaptor molecule (TRAM) (Kawai and Akira, 2009).

The recognition of viral components by TLRs via the TRIF-dependent pathway commonly induces production of type I IFN that can activate target cells in both autocrine and paracrine manners. This includes stimulation with TLR3, TLR4, TLR7, and TLR9 ligands, but not the TLR2 ligand (Kawai and Akira, 2009).

In case of the hepatitis C virus, TLRs such as TLR2 and TLR4 can be induced by the virus (Chang et al., 2007; Machida et al., 2006) and intracellular TLRs such as TLR3 and TLR7 can sense virus RNA (Dolganiuc et al., 2004; Zhang et al., 2016). Activation of TLR3 inhibits HCV replication, suggesting that TLR3 is part of the antiviral response to the infection (Liang et al., 2018; Wang et al., 2009). TLR3 signals are transduced through TIR domain containing adapter-inducing IFN-β (TRIF) leading to activation of the transcription factors IRF3 and NFκB for induction of innate immunity (Dansako et al., 2013; Seki and Brenner, 2008). On the other hand, recognition of the hepatitis C virus by TLR7 in hepatocytes and Kupffer cells leads to production of IFN, or activation of inflammasome pathway (Negash et al., 2013).

1.2. Recognition of viruses by RLR

The RLR (RIG-I like receptors) family includes different receptors such as MDA5 (melanoma differentiation-associated protein 5), LGP2 (Laboratory of Genetics and Physiology 2) and RIG-I which is the best described sensor protein for viral infections (Saito et al., 2007). RIG-I is a cytosolic dsRNA helicase that is encoded in humans by the DDX58 gene. It consists of two N-terminal CARDs (caspase activation and recruitment domains) followed by a central DExD/H box helicase and C-terminal regulatory domains (helicase-CTD) (Ferrage et al., 2012; Yoneyama et al., 2004). Viral dsRNA is sensed by RIG-I via its helicase domain whereas the CARD domains are responsible for downstream signaling (Saito et al., 2007). Upon binding of PAMPs to RIG-I, conformational changes are induced where the C-terminal repression is relieved, followed by binding to the viral RNA and scanning for particular U/UC sequences (Rehwinkel and Reis e Sousa, 2010). Then RIG-I releases CARD domains for ubiquitination by TRIM25 and interaction with 14-3-3ε (a family of regulatory molecules that interact with several signaling proteins) to promote association with intracellular membranes for translocation to the mitochondrial-associated endoplasmic reticulum membrane (mitochondrial-associated membrane, MAM) to recruit a mitochondrial antiviral signaling protein (MAVS), termed RIG-I signaling adaptor protein (Yoneyama et al., 2004; Rehwinkel and Reis e Sousa, 2010). This interaction will in turn activate effector molecules, including the transcription factors IRF3 and NFκB, to drive downstream innate immune signaling (Saito et al., 2007; Yoneyama et al., 2004).

1.3. Recognition of viruses by NLRs

The most recently identified pathway in the recognition of RNA viruses is governed by Nod (nucleotide oligomerization domain)-like receptors (NLRs) that recognize PAMPs, as well as host-derived danger associated molecular patterns (DAMPs) (Schroder and Tschopp, 2010) and cause subsequent activation of inflammasomes (Horvath et al., 2011). Unlike TLRs, this family consists of soluble proteins that survey the cytoplasm for signals that advertise the presence of intracellular invaders (Martinon and Tschopp, 2005). Structurally, NLRs are multi-domain proteins with a tripartite architecture containing a C-terminal region characterized by a series of leucine-rich repeats (Bella et al., 2008), a central nucleotide domain termed the NACHT domain (also referred to as NOD domain) (Kufer et al., 2005), and an N-terminal caspase recruitment (CARD) or pyrin (PYD) domain (Johnson and Gale, 2006). LRRs are believed to function in ligand sensing and auto-regulation, whereas CARD and PYD domains mediate homotypic protein-protein interactions for downstream signaling. NLR subfamilies differ in their N-terminal effector domains, which mediate signal transduction to downstream targets, leading to activation of inflammatory caspases by inflammasomes or NF-κB by NOD signalosomes (Johnson and Gale, 2006). The NACHT domain, which is the only domain common to all NLR family members, enables activation of the signaling complex via ATP-dependent oligomerization (Kufer et al., 2005).

2. Viroporins and their role in the virus life cycle

Since the M2 protein of influenza virus A was described to have ion channel activity in 1992 (Pinto et al., 1992), several ion channel proteins encoded by viruses have been discovered. This new family of virus proteins that alter membrane permeability was named viroporins. Typically, viroporins are comprised of 50–120 amino acids and tend to form homo-oligomers (Scott and Griffin, 2015; Wang et al., 2011). Oligomerization produces a hydrophilic pore which allows ion transport across host cell membranes. Activity of these viral ion channel proteins is involved in many stages of the virus infection cycle. Viroporins play important roles in virus genome replication and assembly, as well as virus particle entry and release from infected cells (Wozniak

Table 1
Summary of viroporins and their proposed functions in the virus life cycle.

Virus	Viroporin	Function	Role of viroporin in activation of NLRP3	References
Influenza A virus (IAV)	M2	In endosomes, it imports hydrogen ions (H^+) into the virions and helps to release viral ribonucleocapsid to the cytosol.	Ion channel activity of M2 enables H^+ export from acidified Golgi, and such activity provides the second signal required for the activation of NLRP3.	(Nieva et al., 2012; Wang et al., 1994; Ichinohe et al., 2010; Sakaguchi et al., 1996; Allen et al., 2009; Chizhmakov et al., 1996; Holsinger et al., 1994; Madan et al., 2008; Pinto and Lamb, 2006; Wang et al., 1993)
		Neutralizes the trans-Golgi network pH and prevents hemagglutinin from becoming fusogenic.	M2 affects ROS production and K^+ efflux which affects IL-1β production and can be blocked by high concentration of extracellular K^+ or by adding ROS inhibitors.	
Human encephalo-myocarditis virus (EMCV)	2B	Modification of intracellular membrane structures.	Manipulates Ca^{2+} homeostasis, stimulating Ca^{2+} flux from intracellular storages to the cytosol, providing second signal for NLRP3 activation and IL-1β production.	(Nieva et al., 2012; Liu et al., 2004; Ito et al., 2012; Carocci et al., 2011; Rajan et al., 2011)
Rhino virus	2B	Forms homomultimers that create pores in ER and Golgi complex membranes, thereby reducing the levels of Ca^{2+} and H^+ in the lumens of these organelles in infected cells.	IL-1β was not inhibited by inhibitors of mitochondrial ROS and cathepsin B, which effectively blocks ATP- and Alum-induced IL-1β secretion	(Nieva et al., 2012; Triantafilou et al., 2013a)
Picorna virus	2B	Individual expression of 2B results in inhibition of protein trafficking through the Golgi complex.		(Nieva et al., 2012; Madan et al., 2008)
Polio virus	2B	2B mutations cause early defects in viral RNA replication. May be required for the activity of the precursor 2BC to accumulate membranous replication vesicles. Can induce apoptosis.		(Aldabe et al., 1996; Agirre et al., 2002)
Human immunodeficiency virus (HIV)	VPU	Required for budding of the virus	An association between NLRP3 single nucleotide polymorphisms (SNP) and susceptibility to HIV infection has been reported, although the exact mechanism remains unknown.	(Dube et al., 2010; Gonzalez and Carrasco, 2003; Liu et al., 2004; Madan et al., 2008; Schubert et al., 1996)
Respiratory syncytial virus (RSV)	SH	SH gets localized in the cell membranes and intracellular organelle membranes, and changes permeability by disrupting membrane architecture. SH protein is important for viral infectivity, its exact role during viral infection is not clear. Some studies suggest an ancillary role in virus-mediated cell fusion.	Accumulates in the Golgi network within lipid raft structures, forming ion channels selective for monovalent cations (Na^+ and K^+), which triggers the translocation of NLRP3 from the cytoplasm to the Golgi network and its subsequent activation.	(Segovia et al., 2012; Triantafilou et al., 2013b; Bukreyev et al., 1997; Simoes, 1999; Wilkinson et al., 2006)
Corona virus	E protein	E protein generates an oligomeric structure that forms an ion-conductive pore in planar lipid bilayers.	Causes cations imbalances that can be sensed by NLRP3 inflammasome	(An et al., 1999; Jiang et al., 2005; Verdia-Baguena et al., 2012)
	3a protein	3a protein modulates virus release; however, this protein is not essential for virus viability.		
Classical swine fever virus (CSFV)	P7	It is involved in viral virulence in swine, pore formation and modification of Ca^{2+} membrane permeability.	Disruption of Ca^{2+} homeostasis	(Gladue et al., 2012)
Hepatitis C virus (HCV)	P7	It is needed for polyprotein processing and is essential for a late step in viral assembly and release of infectious virions. Plays an essential role in the production of virus particles. Dissipate the proton gradient of the acidic vesicular compartments, causing leakage of protons into the cytosol. This inhibition of acidification is required for the production of virus particles.	Disruption of H^+, K^+, and $Ca2^+$ homeostasis across intracellular vesicles provides signal 2 for NLRP3 activation.	(Nieva et al., 2012; Steinmann et al., 2007; Pavlovic et al., 2003; OuYang et al., 2013; StGelais et al., 2009; Premkumar et al., 2004; Boson et al., 2011; Clarke et al., 2006; Cook and Opella, 2010; Cook et al., 2011; Haqshenas et al., 2007; Khaliq et al., 2011; Li et al., 2012; Sakai et al., 2003; StGelais et al., 2007; Wang et al., 2013)

Table 2
Structure and activation pathway of biologically important inflammasomes.

Inflammasome	Structure	Pathway of activation	References
NLRP1/NALP1b	Widely expressed in different cells, it has a C-terminal extension that harbors exclusively an FIIND motif and a CARD, which was shown to recruit caspase-5 or a second caspase-1. N-terminal PYD interacts with PYD of ASC whose CARD can recruit pro-caspase-1.	Two steps activation process involving primary activation by microbial ligands followed by rNTP binding to the nucleotide binding domain of NLRP1. It is also involved in inflammasomes response to anthrax lethal toxin	(Tschopp et al., 2003; Faustin et al., 2007; Kummer et al., 2007)
NLRP3/NALP3	Found mainly in immune cells, epithelial cells, and osteoblasts. The core structure of the NALP3 inflammasome is formed by NALP3, the adaptor ASC, and caspase-1. PYD-PYD and CARD-CARD homotypic interactions are crucial for the recruitment and activation of either the adaptor ASC or the inflammatory caspases. It does not have the NALP1 C-terminal extension; instead, CARDINAL (a protein very similar to the NALP1 C terminus) interacts with other inflammasomes.	RNA viruses activate NLRP3 either via RNA-sensing kinase PKR or other signals such as viroporins mediated ionic disturbance, ROS, uric acid. NALP3 also selectively binds ATP/dATP, and nucleotide binding is necessary for oligomerization of the NACHT domain. SGT1-HSP90 complex binds the NALP3 LRR domain	(Martinon and Tschopp, 2005; Martinon et al., 2007; Kummer et al., 2007; Martinon and Tschopp, 2007; Mayor et al., 2007)
NLRC4/IPAF	Expressed in brain, macrophages and myeloid cells-rich tissues such as spleen, lung, and liver. It contains an N-terminal CARD, a central NACHT domain and C-terminal LRRs.	NLRC4/IPAF forms an inflammasome that activates caspase-1 in response to flagellin monomers in an ASC-independent manner, independent of TLR5.	(Martinon and Tschopp, 2005; Martinon et al., 2007; Martinon and Tschopp, 2007; Miao et al., 2006; Poyet et al., 2001)
AIM2	It has N-terminal DAPIN (pyrin) domain and C-terminal HIN-200 domain which has two oligonucleotide binding folds.	AIM2 induces caspase-1 activation upon sensing nucleic acids, has been suggested to be critical for the activation of host defense against vaccinia virus and Francisella tularensis. C terminal of HIN domain can bind to dsDNA leading to oligomerization of inflammasome complex.	(Hornung et al., 2009; Fernandes-Alnemri et al., 2009)

et al., 2010). Some studies reported that the deletion of viroporin-encoding genes from virus genome significantly reduces the formation of virus progeny, and thus lowers virus pathogenicity. This highlights the essential role of viroporins in the viral life cycle, opening a new window for their potential use as antiviral targets (Nieva et al., 2012; Steinmann et al., 2007; Steinmann and Pietschmann, 2010).

The study of viroporins initially focused on M2 of the influenza A virus (IAV), picornavirus protein 2B (P2B) and togavirus protein 6 K, while only recently HIV-1 viral protein U (Vpu) and HCV p7 were included (Nieva et al., 2012). A summary of viroporins and their roles in viral life cycle is provided in Table 1. According to the number of transmembrane domains and their topology, viroporins are divided into two classes. Class I viroporins possess a single membrane-spanning domain. These viroporins contain proteins that are inserted into the membrane with either a luminal amino terminus and cytosolic carboxyl terminus (class IA) such as M2 of IAV, Vpu of HIV-1 and E protein of coronavirus, or a cytosolic amino terminus and lumenal carboxyl terminus (class IB) such as small hydrophobic protein of human respiratory syncytial virus (RSV) and P3A of polio virus.

Class II viroporins possess two transmembrane domains that are connected by a loop of basic amino acids. The N and C termini of class IIA viroporins both face the ER lumen such as p7 of HCV and 6 K of sindbis virus, whilst in class IIB viroporins the opposite orientation is found, with both termini located in the cytosol as seen with P2B of polio virus (Brohm et al., 2009; Pavlovic et al., 2003; Stouffer et al., 2008).

Along the study of the nature of viroporins, the question has been raised of whether ion gated channels or small sized pores are formed and whether ions are redistributed according to their concentration gradient (OuYang et al., 2013; StGelais et al., 2009). Studies of transgenically expressed viroporins in cell cultures, planar lipid bilayer or liposomes have shown that each viroporin has its individual channel function, which determines how it would affect membrane permeability (Nieva et al., 2012). For instance, the most characterized viroporin M2 was studied by patch-clamp recordings and proton conductance was demonstrated (Chizhmakov et al., 2003). The channel was shown to conduct protons selectively across cellular membranes which causes loss of organelle acidification (Wang et al., 1994). The same activity was confirmed by biochemical assays such as haemagglutinin protein translocation to the cell surface. which is a direct consequence of exposure to increasing pH in vesicles by M2 activity (Alvarado-Facundo et al., 2015; Ciampor et al., 1992; Griffin et al., 2004). Similarly, Vpu of HIV-1 was shown to be a voltage-gated proton selective channel in electrophysiological studies (Dube et al., 2010; Gazina and Petrou, 2012). A cation selective behavior has been reported for HCV p7 (Griffin et al., 2003; Premkumar et al., 2004), togavirus 6 K and the notorious coronavirus envelope small membrane protein (E); causative agent of SARS, MERS and the current outbreak COVID-19 (Lu et al., 2006). HCV p7 was shown to be a pH-gated proton channel with genotype-dependent specific sensitivity to channel blocking drugs (Breitinger et al., 2016). Another reported activity of viroporins is the alteration of intracellular calcium homeostasis where entry of extracellular calcium and/or Ca^{2+} leakage from intracellular stores, such as the mitochondria, ER and Golgi complex has been observed (Triantafilou et al., 2013a). Certain viroporins, such as P2B, polyprotein P2BC and P3A from poliovirus (Aldabe et al., 1996) or envelope small membrane protein (E) from coronavirus induce intracellular membrane remodeling to generate new membrane vesicles (called the viroplasm) that serve as viral replication sites (Lu et al., 2006; Agirre et al., 2002). Several studies have claimed the involvement of viroporins in virus induced programmed cell death such as the case with coronavirus E protein and picornavirus 2B viroporin (An et al., 1999). This was attributed to perturbation of ion homeostasis, a common hallmark of apoptosis, which causes depolarization of the plasma membrane associated with intracellular cations overload and cell volume decreases as a result of anions and H_2O efflux (Gonzalez and Carrasco, 2003; Ueda et al., 2010).

3. Inflammasomes

Although the biochemistry and diversity of inflammasomes are still poorly understood, four inflammasomes have been identified and defined by their NLR protein; the NLRP1/NALP1b inflammasomes, the NLRC4/IPAF inflammasome, the NLRP3/NALP3 inflammasome and the AIM2 (absent in melanoma) containing inflammasome (Tschopp et al., 2003). A summary of the four prototypes of inflammasomes is provided in Table 2.

Inflammasomes are molecular platforms activated upon cellular infection or stress that trigger the maturation of proinflammatory

cytokines such as IL-1β to engage innate immune defenses (Latz et al., 2013). They are multi-protein complexes made up of a sensor protein, the adaptor protein ASC, and the cellular protease caspase-1 (Schroder and Tschopp, 2010). Caspases are cysteine proteases that initiate or execute cellular programs, leading to inflammation or cell death. Inflammasomes activate a class of caspases known as inflammatory caspases (Martinon et al., 2007). Inflammatory caspases in mammals have a CARD domain followed by a domain containing the catalytic residue cysteine (Boatright and Salvesen, 2003; Martinon et al., 2002). Caspase-1 itself is synthesized as an inactive 45 kDa zymogen (pro-caspase-1) that undergoes autocatalytic processing following an appropriate stimulus (Boatright and Salvesen, 2003). It is activated within the inflammasome multiprotein complex via interaction with ASC (apoptosis-associated speck-like protein containing a carboxy-terminal CARD), a bipartite adapter protein that bridges NLRs and caspase-1 (Nadiri et al., 2006).

The proinflammatory cytokines of the IL-1 family, most notably IL-1β and IL-18 play very important roles in antimicrobial host defense (Dinarello, 2009). IL-1β activates the release of other proinflammatory cytokines such as TNF and IL-6, and induce a Th17 bias in the cellular adaptive responses as shown in Fig. 1 (Davis et al., 2011). It is also responsible for the acute phase response, which includes fever, acute protein synthesis, anorexia, and somnolence. and coordinates programs as diverse as cellular recruitment to the site of infection or injury and regulation of sleep, appetite, and body temperature (Martinon et al., 2009). IL-18 is another member of the IL-1 family that acts as an important regulator of innate and acquired immune responses. It is produced during chronic inflammation, in autoimmune diseases, in a variety of cancers, and in the context of numerous infectious diseases (Gracie et al., 2003). IL-18 induces the production of multiple cytokines including IFN-γ, IL-13, IL-4, IL-8 as well as both Th1 and Th2 lymphokines, and is an important stimulator of antigen-activated Th1 cells (Shereck et al., 2012). Through these mechanisms, cytokines of the IL-1 family are a crucial component of the host defense (Dinarello, 2009, 1984).

Proinflammatory stimuli induce expression of the inactive IL-1β and IL-18 proforms, but cytokine maturation and release are controlled by inflammasomes (Martinon et al., 2002, 2009). It is now generally accepted that activation and release of IL-1β requires two distinct signals. The nature of these signals *in-vivo* during infection or inflammation is not completely defined (Negash et al., 2013; Latz et al., 2013; Yang et al., 2019). However, *in-vitro* studies showed that the first signal can be triggered by various PAMPs and DAMPs following toll-like receptor (TLR) activation, which induces the synthesis of pro IL-1β (Negash et al., 2013; Pang and Iwasaki, 2011). The second signal is triggered by different DAMPS and PAPMS promoting NLRP3 inflammasome assembly and caspase-1-mediated activation of pro IL-1β and pro IL-18. The requirement for a second signal for IL-1β maturation might constitute a fail-safe mechanism to ensure that the induction of potent inflammatory responses occurs only in the presence of a bona fide stimulus, such as pathogen infection and/or tissue injury (Christgen and Kanneganti, 2019). Signals required for activation and secretion of IL-1β and IL-18 are summarized in Fig. 2.

4. Mechanism of activation of NLRP3

NLRP3 is activated upon exposure to whole pathogens, as well as a number of structurally diverse PAMPs, DAMPs, and environmental irritants (Martinon et al., 2009). A number of host-derived molecules, which are indicative of injury, activate the NLRP3 inflammasome. ATP and certain bacterial toxins, such as nigericin and maitotoxin, cause a change in the intracellular ion composition leading to the activation of the NLRP3 inflammasome (Muruve et al., 2008). The NLRP3 inflammasome also detects signs of metabolic stress, including elevated extracellular glucose and monosodium urate (MSU) crystals. Three models have been proposed to explain the mechanisms leading to NLRP3 activation; the first model adopts pore formation in cellular membrane as a mechanism that allows extracellular NLRP3 agonists to access the cytosol and directly interact with NLRP3 (Kanneganti et al., 2007). A second model was proposed for activators that form crystalline or particulate structures, such as silica, asbestos, amyloid β, and alum, wherein engulfment of these agonists by phagocytes leads to lysosomal damage, resulting in cytosolic release of lysosomal contents that are somehow sensed by the NLRP3 inflammasome (Halle et al., 2008; Hornung et al., 2008). According to a third model, all NLRP3 agonists trigger the generation of reactive Oxygen species (ROS), and this common pathway engages the NLRP3 inflammasome (Cruz et al., 2007). The production of ROS represents one of the most evolutionarily conserved pathways of response to infection or injury. The source of ROS is under debate where some studies claim one or several NADPH oxidases to be involved, while others support mitochondrial origin (Cruz et al., 2007). A recent report associates a ROS-sensitive NLRP3 ligand, thioredoxin-interacting protein (TXNIP/VDUP1), in NLRP3 activation (Xiao et al., 2016).

5. How are viroporins linked to inflammasomes activation?

Inflammation has always been associated with viral infections, raising the question of which viral PAMPs are responsible for induction of inflammatory response (Tschopp et al., 2003). Recently several studies have linked activation of NLRP3 inflammasomes and production of the inflammatory cytokine IL-1β to viral infections such as influenza A virus (IAV), hepatitis C virus (HCV) and encephalomyocarditis virus (EMCV) (Farag et al., 2017; Ichinohe et al., 2010; Liu et al., 2004) as summarized in Fig. 3.

5.1. Influenza A virus: M2 viroporin

Ichinohe et al. investigated the mechanism by which influenza virus activates the NLRP3 inflammasome pathway (Ichinohe et al., 2010). TLR7 of dendritic cells recognizes influenza genomic RNA, providing signal 1 that is required for the transcription and synthesis of pro IL-1β and pro IL-18 (Ichinohe et al., 2010). The second signal is provided by M2 ion channel activity across the trans-Golgi network (TGN) (Sakaguchi et al., 1996). M2 protein was reported to assist in influenza viral entry into the cell by equilibrating the pH of the virus within the acidifying endosome. This step crucial to facilitate replication of the viral genome by disruption of the interactions between the viral ribonucleoprotein (vRNP) complex and the matrix protein, M1, which coats the vRNP (Wang et al., 1994). In addition, the M2 channel allows protons from the acidic TGN to neutralize the pH of its lumen in order to prevent the premature maturation of hemagglutinin to its low-pH fusogenic form (Ichinohe et al., 2010; Sakaguchi et al., 1996).

By altering ionic concentrations of intracellular compartments, M2 can trigger NLRP3 inflammasome activation (Ichinohe et al., 2010; Kuriakose and Kanneganti, 2017). It was shown that the M2del29-31 mutant of the influenza virus (lacking amino acids 29–31 from the transmembrane region of the M2 protein) completely failed to stimulate inflammasome activation and release of IL-1β or IL-18 from BMM and BMDCs (Ichinohe et al., 2010). In addition, mature caspase-1 and IL-1β were only detected in the supernatants of cells infected with wild-type influenza, but not M2del29-31 mutant virus, despite the fact that M2del29-31 mutant was capable of infecting target cells to an extent comparable to the wild-type virus. M2del29-31 mutant virus-infected cells also expressed comparable amounts of pro IL-1β mRNA (Ichinohe et al., 2010). It was also shown that bone marrow derived macrophages (BMMs) and bone marrow derived dendritic cells (BMDCs), stimulated with LPS (signal 1) and transduced with a lentivirus expressing the M2 protein derived from H1N1, were capable of producing both IL-1β and IL-18 compared to controls. In unprimed BMM, however, M2 expression alone was not sufficient to trigger IL-1β release. This confirms the hypothesis that pathogen-encoded ion channels, in addition to more

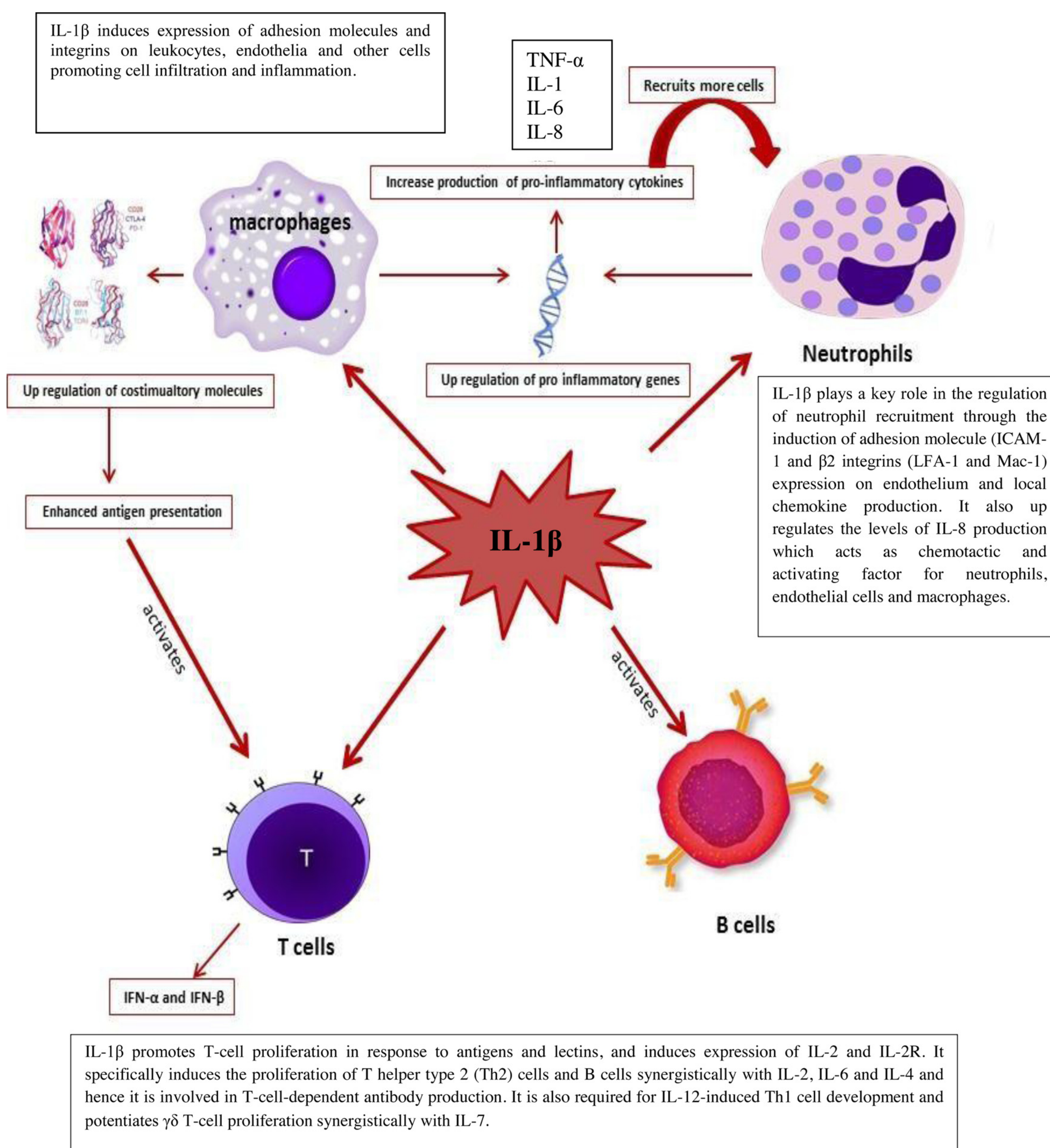

Fig. 1. The role of IL-1β in eliciting immune responses against infections. IL-1β up-regulates a broad range of proinflammatory activities in immune cells. It induces rapid recruitment of neutrophils to sites of infection, activation of the endothelial adhesion molecules and induction of chemokines. It also induces the release of many cytokines such as IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IFN-γ, TNF, and PDGF. It also induces proliferation of T-helper and B cells and enhances antigen presentation.

drastic disruption of membranes by pore-forming toxins or membrane rupture, can trigger signal 2 for NLRP3 inflammasome activation (Ichinohe et al., 2010). In a different study, it was reported that M2-dependent IL-1β production of influenza virus could be blocked by high concentration of extracellular K^+ or by treating cells with ROS inhibitors (Allen et al., 2009). M2 and NLRP3 dependent IL-1β and IL-18 secretion also occurs in human dendritic cells infected with influenza virus (Fernandez et al., 2016).

5.2. Respiratory syncial virus (RSV): SH viroporin

A recent study (Segovia et al., 2012) showed that infection with RSV triggers NLRP3 activation via reactive oxygen species (ROS) production, where elevated levels of IL-1β were produced by RSV-infected airway cells. RSV can activate NLRP3/ASC inflammasome via activation of the TLR2/MyD88 (myeloid differentiation primary response 88)/nuclear factor kB (NF-kB) pathway for pro IL-1β synthesis. The second signal, however, was shown to be provided by a small hydrophobic (SH) viroporin encoded by RSV (Segovia et al., 2012; Ravi Kumar et al., 2018; Shil et al., 2018). Another study by Triantafilou et al. (2013b) confirmed these findings and showed that SH viroporin accumulates in the Golgi within lipid raft structures, forming ion channels selective for monovalent cations (Na^+ and K^+). This triggers the translocation of NLRP3 from the cytoplasm to the Golgi and its activation. Moreover an SH deletion mutant RSV rgRSV-GF (ΔSHRSV lacking the viroporin SH) can grow and replicate efficiently in cell culture but is unable to trigger inflammasome activation or stimulate the production of IL-1β Triantafilou et al. (2013b). However, in the same study, IL-6 a non-inflammasome cytokine, as well as IFNβ and pro IL-1β were produced normally Triantafilou et al. (2013b). Several viroporin inhibitors were also examined in RSV infected cellular models. HMA and Benzamil were successful in inhibiting IL-1β secretion upon RSV infection (Triantafilou et al., 2013a; Triantafilou et al. (2013b).

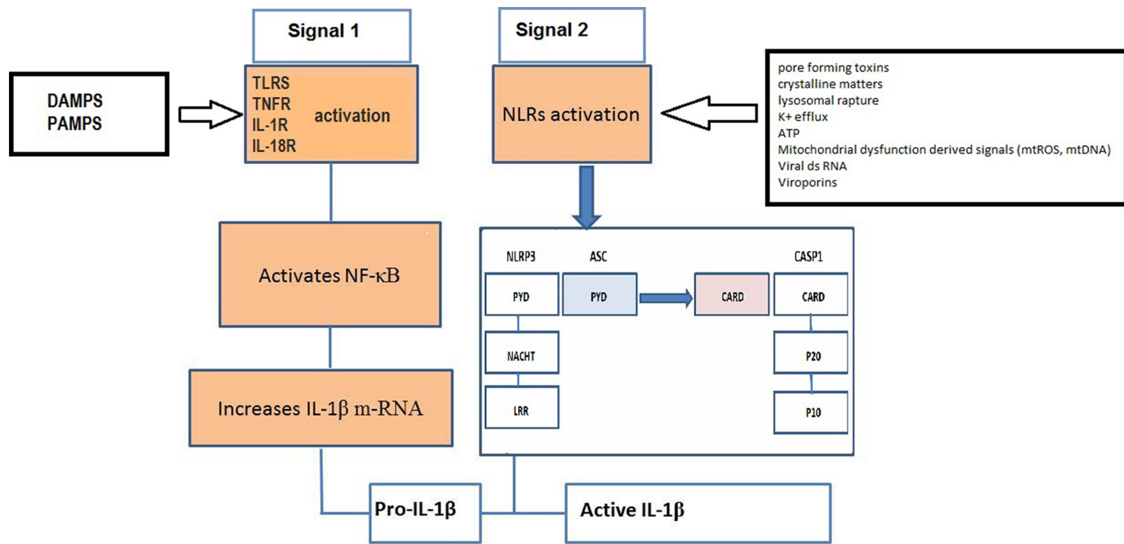

Fig. 2. Signals required for activation and release of IL-1β and IL-18. The first signal can be triggered by various PAMPs or DAMPS recognized by Toll-like receptor (TLR), IL-1 receptor (IL-1R), IL-18 receptor (IL-18R) or tumor necrosis factor receptor (TNFR). The activation of such receptors leads to the activation of NF-κB which induces the synthesis of pro-IL-1β. The second signal is provided by the activation of the inflammasome complex and caspase-1 leading to IL-1β processing. NLRP3 inflammasome detects signs of metabolic stress, including elevated extracellular glucose, monosodium urate (MSU) crystals, ATP and changes in the intracellular ion composition caused by viral encoded ion channels; viroporins activity and certain bacterial toxins, such as nigericin and maitotoxi. NLRP3 oligomerization leads to PYD domain clustering and presentation for homotypic interaction with the PYD- and CARD-containing adaptor ASC, whose CARD domain in turn recruits the CARD of procaspase-1. Procaspase-1 clustering permits autocleavage and formation of the active caspase-1 p10/p20 tetramer. Caspase-1 is activated within the inflammasome multiprotein complex through interaction with ASC (apoptosis-associated speck-like protein containing a carboxy-terminal CARD), a bipartite adapter protein that bridges NLRs and caspase-1.

5.3. Rhinovirus: 2B viroporin

Inflammasome activation was also studied in coronavirus and rhinovirus, both of which possess viroporins. Studies have shown elevated levels of inflammatory cytokines such as IL-1β in both infections (Davis et al., 2011). A study by Dolinay et al. (Dolinay et al., 2012) showed that inflammasome-regulated cytokines IL-18 and IL-1β are crucial mediators of acute lung injury in mouse models, implicating the important role of inflammasome pathway and its downstream cytokines in respiratory inflammation (Dolinay et al., 2012).

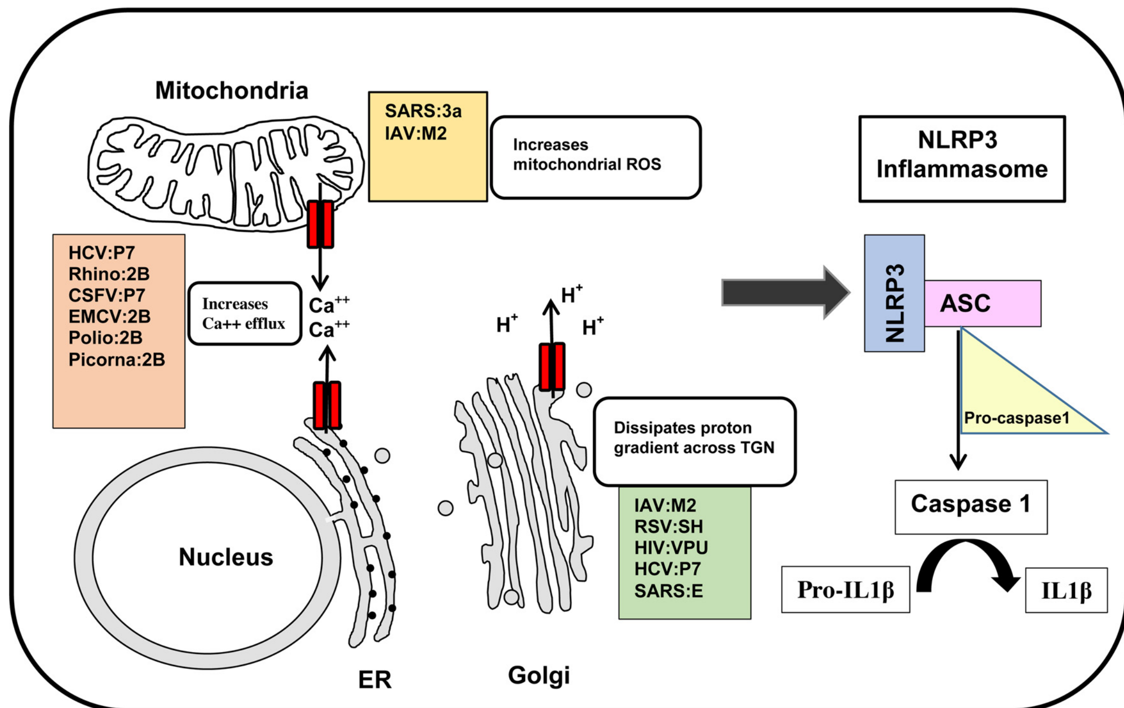

Fig. 3. Viroporins activity and activation of inflammasomes. Viroporins activities can be clustered into three main groups that have been linked to activation of NLRP3 inflammasomes. The first group of viroporins pumps protons and dissipates proton gradient across trans-golgi network, eg.M2 of influenza A virus. The second group manipulates Ca^{2+} homeostasis, stimulating Ca^{2+} flux from intracellular storages to the cytosol providing the second signal for NLRP3 activation and IL-1β production such as 2B of polio and rhino virus. The third group increases mitochondrial stress and affects ROS production such as 3a of corona virus.

In rhinovirus infection, activation of NLRP3 and NLRC5 was reported in several studies (Triantafilou et al., 2013a; Chen and Ichinohe, 2015; Radzikowska et al., 2017). Rhinovirus viroporin 2B forms membrane channels that manipulate Ca^{2+} homeostasis where it increases cytosolic Ca^{2+} by reducing ER and Golgi Ca^{2+} levels (Triantafilou et al., 2013a; Chami et al., 2006). Triantafilou, et al. have shown that Rhinovirus-induced calcium flux triggers NLRP3 and NLRC5 activation in bronchial cells and such activity can be blocked by both Ca^{2+} channel inhibitor verapamil and Ca^{2+} chelator BAPTA-AM [acetoxymethyl ester derivative of BAPTA, bis(aminophenoxy)ethane-tetraacetic acid] (Triantafilou et al., 2013a).

5.4. Coronavirus: E and 3a viroporins

As for corona virus infection (SARS, MERS and COVID-19), studies have reported higher IL-18 and IL-1β levels, not only in the blood of the patients, but also in lungs and lymphoid tissues, indicating increased inflammasome activation (Chen et al., 2019; Clay et al., 2014; Triantafilou and Triantafilou, 2014). Corona virus encodes two viroporins; E protein and 3a protein, which act as ion-conductive pores in planar lipid bilayers and are required for maximal SARS-CoV replication and virulence. They could possibly be responsible for inflammasome activation (Lu et al., 2006; Castano-Rodriguez et al., 2018). It was recently shown that the SARS 3a protein activates the NLRP3 inflammasome in lipopolysaccharide-primed macrophages by affecting K^+ efflux and mitochondrial reactive oxygen species (Chen et al., 2019). Another study showed that the SARS-CoV open reading frame 3a (ORF3a) accessory protein activates the NLRP3 inflammasome by promoting TNF receptor-associated factor 3 (TRAF3)–mediated ubiquitination of apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) (Siu et al., 2019).

5.5. Classical swine fever virus: P7 viroporin

One study investigated the production of IL-1β from macrophages following classical swine fever virus (CSFV) infection (Gladue et al., 2012). Indeed, after CSFV infection IL-1β was up-regulated through caspase-1 activation. Furthermore, CSFV viroporin p7 protein (a viroporin involved in modification of Ca^{2+} membrane permeability) induces IL-1β secretion which can be inhibited by the ion channel blocker amantadine (Fung et al., 2015a; Largo et al., 2016a) (Fung et al., 2015b; Largo et al., 2014, 2018; Largo et al., 2016b).

The hypothesis that p7 activity is involved in CSFV-mediated IL-1β production was supported in one study where macrophages 3D4/2 were transfected with either plasmids pEGFP-p7 or pEGFP-C3 (Lin et al., 2014). Increased expression of both IL-1β mRNA and secretion of IL-1β in p7 expressing cells was reported (Lin et al., 2014).

5.6. Encephalomyocarditis virus: 2B viroporin

In a study of encephalomyocarditis virus (EMCV) activity, Ito et al. (2012) reported that the virus activates the NLRP3 inflammasome in mouse dendritic cells and macrophages. Transfection with RNA from EMCV virions or EMCV-infected cells induced robust expression of type I interferons in macrophages, however it failed to stimulate secretion of IL-1β Ito et al. (2012). On the other hand, the EMCV viroporin 2B was sufficient to induce inflammasome activation in LPS-primed macrophages. While untransfected cells or those transfected with the gene encoding the EMCV non-structural protein 2A or 2C expressed NLRP3 uniformly throughout the cytoplasm, NLRP3 was redistributed to the perinuclear space only in cells transfected with the gene encoding the EMCV 2B or influenza virus M2 protein Ito et al. (2012). Ca^{2+} flux from intracellular storages to the cytosol can be the mechanism by which EMCV activates the NLRP3 inflammasome, since IL-1β production was not affected by inhibitors of mitochondrial ROS and cathepsin B, which effectively blocked ATP and alum-induced IL-1β secretion, respectively Ito et al. (2012). Another member of the picornaviridae family is foot-and-mouth disease virus (FMDV). Both FMDV RNA and viroporin 2B were shown to stimulate NLRP3 inflammasome activation by elevation of intracellular ion, but not dependent on mitochondrial reactive oxygen species (ROS) and lysosomal cathepsin B (Zhi et al., 2020).

5.7. Hepatitis C virus: P7 viroporin

HCV p7 is a small hydrophobic viroporin of 63 amino acids (Carrere-Kremer et al., 2002). P7 was reported to substitute for the well-characterized M2 vioporin of Influenza (Griffin et al., 2004) where it was able to dissipate the proton gradient across exocytic vesicles as well as the TGN to prevent acid induced premature fusogenic conformational changes of multi basic haemagglutinin (Ciampor et al., 1992). The ion channel was also reported to cause global loss of organelle acidity upon expression (Wozniak et al., 2010). Reports have shown that IL-1β levels are higher in HCV induced liver diseases than in other forms of liver damage, however the mechanism by which HCV infection activates NLRP3 is still under investigation (Negash et al., 2013; Farag et al., 2017; Negash et al., 2019). Monocyte-derived human macrophages (THP-1) incubated with cell culture-grown HCV enhance the secretion of IL-1β/IL-18 into culture supernatants. A similar cytokine release was also observed for peripheral blood mononuclear cell (PBMC)-derived primary human macrophages and Kupffer cells (liver-resident macrophages) upon incubation with HCV. THP-1 cells incubated with HCV led to caspase-1 activation and release of proinflammatory cytokines. Subsequent studies demonstrated that HCV induces pro IL-1β and pro IL-18 synthesis via the NF-κB signaling pathway in macrophages. Furthermore, introduction of HCV viroporin p7 RNA into THP-1 cells was sufficient to cause IL-1β secretion (Shrivastava et al., 2013).

When p7 constructs of different genotypes were expressed in human embryonic kidney (HEK-293) cells, as well as in murine macrophage (RAW 264.7) cells (Breitinger et al., 2016; Farag et al., 2017), the effects of extracellular concentrations of H^+ concentration and the channel blocker rimantadine on cytokine production were demonstrated (Farag et al., 2017). In LPS-primed macrophages, p7 increased levels of IL-1β in a genotype dependent manner. Treatment of transfected cells with rimantadine abolished p7-induced effects completely, in agreement with the pH-response profile of p7 channels, concluding that p7 with its proton conductance activity can provide the second signal required for inflammasome activation (Farag et al., 2017, 2018).

In another report (Negash et al., 2013), the hepatic macrophage/HCV interface and IL-1β production through the NLRP3 inflammasomes was revealed. Following virus uptake in macrophages, HCV proteins are transiently produced but then decay. HCV proteins have been shown to stimulate ROS accumulation and regulate ion efflux (Negash et al., 2019; Quarato et al., 2013; Scrima et al., 2018). Moreover, the HCV p7 protein activity was suggested to impart NLRP3 inflammasome activation during HCV infection by providing the required signal 2 (Negash et al., 2013).

6. Conclusion and future perspective

In the past decade, the NLRP3 inflammasome has been described as an important mediator of virus-induced inflammation. Recent studies have shown that several viruses are able to activate the NLRP3 inflammasome, which in turn induces secretion of proinflammatory cytokines. The pathway has been the center of attention of extensive research and a growing body of evidence suggests perturbation of membrane permeability by viroporins and subsequent disruption of ions homeostasis in cellular compartments as a possible activation signal required for the production of IL-1β and IL-18.

Many viruses encode viroporins which are reported to conduct the flux of different ions (such as H^+ and K^+) across cell membranes, affecting both the concentration of ions inside and outside the cells and

the permeability of the membrane to these ions. Some of the viroporins are also able to target intracellular compartments affecting pH or Ca^{2+} homeostasis. Future study of the exact mechanisms by which NLRP3 detects viruses and its correlation to viroporins is needed to provide us with a better understanding of viral diseases to design effective interventions. Targeting the inflammasome in combination with viroporin inhibitors in virus-induced disease is emerging as an attractive therapeutic target in the therapy of virus-associated diseases.

CRediT authorship contribution statement

N.S. Farag: Writing - original draft, Validation, Investigation. **U. Breitinger:** Writing - review & editing, Conceptualization. **H.G. Breitinger:** Writing - review & editing, Resources, Project administration. **M.A. El Azizi:** Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no competing interests regarding this study.

Acknowledgements

Part of this work was supported by STDF grant 1795 to HGB. All data generated or analyzed during this study are included in this published article.

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