Subverting the mechanisms of cell death: Flavivirus manipulation of host cell responses to infection

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Abbreviations:

BAX Bcl-2-like protein
BAK Bcl-2-homologous killer
TNF-α Tissue necrosis factor alpha
DENV Dengue Virus
JEV Japanese Encephalitis Virus
WNV West Nile Virus
ZIKV Zika Virus
NS Flavivirus non-structural protein
C Flavivirus capsid protein
E Flavivirus envelope protein
M Flavivirus small membrane protein
IL-1β Interleukin one beta
IL-10 Interleukin ten
CASP Caspase, cysteine-aspartic protease
ER Entoplasmic reticulum
ROS Reactive oxygen species
P53 Tumour protein 53
PI31-Akt Phosphatidylinositol 3-kinase/protein kinase B signaling pathway
JAK-STAT Janus kinase – signal transducer and activation of transcription proteins
YFV Yellow Fever Virus
PG Proteoglycan
HMGB1 High mobility group box-1 protein (amphoterin)
HSPG Heparan sulphate proteoglycan
HS Heparan sulphate
CS Chondroitin sulphate
CD-44 A cell surface glycoprotein, also known as HCAM; homing cell adhesion molecule
CHOP Cyclic AMP response element-binding transcription factor homologous protein
RANTES A chemokine; regulation on activation, normal T cell expressed and secreted, also known as CCL5 chemokine (C-C motif) ligand 5.
NDST N-deacetylase and N-sulfotransferase-1
EXT1 Exostosin-1
NPCs Neural progenitor cells

Abstract

Viruses exploit host metabolic and defence machinery for their own replication. The flaviviruses, which include Dengue (DENV), Yellow Fever (YFV), Japanese Encephalitis (JEV), West Nile (WNV) and Zika (ZIKV) viruses, infect a broad range of hosts, cells and tissues. Flaviviruses are largely transmitted by mosquito bites and humans are usually incidental, dead-end hosts, with the notable exceptions of YFV, DENV and ZIKV. Infection by flaviviruses elicits cellular responses including cell death via necrosis, pyroptosis (involving inflammation), or apoptosis (which avoids inflammation). Flaviviruses exploit these mechanisms and subvert them to prolong viral replication. The different effects induced by DENV, WNV, JEV and ZIKV viruses are reviewed. Host cell surface proteoglycans bearing glycosaminoglycan (GAG) polysaccharides - heparan/chondroitin sulphate (HS/CS) - are involved in initial flavivirus attachment but, also during the expression of non-structural viral proteins, and play a role in disease aetiology. Recent work has shown that ZIKV infected cells are protected from cell death by exogenous heparin (a GAG structurally similar to host cell surface HS), raising the possibility of further subtle involvement of HS proteoglycans in flavivirus disease processes. The aim of this review is to synthesize information regarding DENV, WNV, JEV and ZIKV from two areas that are usually treated separately; the response of host cells to infection by flaviviruses and the involvement of cell surface GAGs in responses to those infections.

INTRODUCTION

HOST CELL RESPONSES TO VIRAL INFECTION

A wide group of viruses have life-cycles that involve complex interactions with the metabolic machinery of their hosts. These include the *poxviridae, adenoviridae, retroviridae, picornoviridae, flaviviridae* and *orthomyxoviridae* [1]. Several virus types, including those with large genomes, such as poxviruses and herpesviruses, are known to block host apoptotic pathways [2,3]. One early response by host cells to an invading microorganism is to initiate cell death through several mechanisms (see below). Depending on where the invading virus is detected, this could be initiated, through intracellular signaling systems, such as BAX (Bcl-2 regulator protein). Many other host cell responses are, however, available by which cell death can be influenced, enabling viruses to enhance their survival and replication. These include autophagy, apoptosis and pyroptosis; each of which has distinct mechanisms and outcomes (**Figure 1**);

Autophagy: In autophagy, the cellular constituents are partitioned and fused with lysosomes to allow their degradation and re-cycling [4]. This is a means of extending the longevity of a cell under stress and can be activated by infection. It is a highly conserved surveillance process involving the transport of lipids, proteins and organelles from the cytoplasm into double-membrane vesicles (autophagosomes) and then to lysosomes for degradation [reviewed in [5]. For DENV, JEV and ZIKV but, potentially also other *flaviviridae*, autophagy can be activated, to provide a means by which viral replication can be prolonged and cause pathology [6].

Apoptosis: Apoptosis is a form of cell death which is programmed and highly regulated, during which the cell undergoes a series of morphological changes, mitochondrial stress and formation of apoptotic bodies, allowing the cell contents to be consumed by phagocytes, avoiding any resulting inflammatory response. Apoptosis can be achieved

through the BAX and BAK-mediated intrinsic and TNF-α-mediated extrinsic apoptotic pathways.

Pyroptosis: In contrast, pyroptosis requires the presence of CASP-1 in a complex termed the pyroptome or inflammasome, in each macrophage [7] and involves activation of the inflammasome within macrophages resulting in release of cell debris (DNA, ATP, cytokines & other proteins), thereby sustaining the inflammatory response in the surrounding tissue.

Each of these responses is triggered by activation of distinct pathways and each response leads to distinct consequences (**Figure 1**). From the perspective of the host cell, a pyroptotic response (if it is not overly pronounced; a situation that can lead to a 'cytokine storm') can achieve rapid clearance but, in chronic infections, this leads to sustained inflammation that can also cause uninfected cell death [8]. Infection induces a number of responses, which influence subsequent events, for example, *in vitro* infection of endothelium by flaviviruses increases adhesion and up-regulates major histocompatibility complex molecules, which can be modulated by cytokines [9]. The ultimate outcome of a viral infection, however, depends not only on which routes are activated but, also on the original viral load [4].

The most extensively studied flavivirus in this regard is DENV but, information concerning virus-host (vertebrate and insect vector) interactions other flaviviruses such as WNV, JEV and ZIKV is also emerging. There are fewer data available regarding interactions between many of the flaviviruses and insect tissues or cells. Responses in insects are addressed first and are followed by a survey of the interactions of DENV, WNV, JEV and ZIKV and their consequences for mammalian cells.

RESPONSES TO DENV, WNV, JEV AND ZIKV IN MOSQUITOES

Viruses are introduced into the mosquito through a blood meal from their animal host and replicate in the midgut within a few minutes, then spread through the insect tissue in the haemolymph. The virus is then passed to another animal host during feeding through transmission of infected saliva. [10, 11]. Life-long infection of *Aedes* and *Culex* mosquitoes apparently occurs without pathological effects and viral interference, in which infection with one virus inhibits infection with another, is also observed [12]. For example, *Ae aegypti* infected by DENV-2 and -3 strains simultaneously show higher levels of RNA of the DENV-2 form, but the origins of this phenomenon are not well understood. Viral interference and viral persistence involve the JAK-STAT and Toll pathways of the innate immune system of the insect. Several cellular systems are implicated in flavivirus replication including, for DENV, EF1α, La (translation elongation factor) in replication [13-15]. The immune response in insects is distinct from that in vertebrates. In mosquitoes, this involves three main signaling pathways;

a. Innate immune system, involving the production of antimicrobial peptides, mainly against Gram negative bacteria.

b. The Toll-mediated pathway, against viruses, Gram positive bacteria or fungi and also involves the production of some peptides.

c. Signal transduction through the JAK-STAT pathway, against viruses, particularly in *Aedes*.

The innate response involves the activation of two proteolytic cascades following infection, leading to blood clotting and melanization, resulting in release of reactive oxygen species. Phagocytosis of bacteria and encapsulation of larger parasites by blood cells can also occur. For virus infection, the Toll and JAK-STAT pathways are the most important and have been studied most extensively for DENV in *Aedes aegypti* [16-20]. The flavivirus genome comprises a single open-reading frame, which encodes a total of 10 viral proteins (**Figure 2**). Several molecular determinants have been identified, which contribute to the infection of mosquito cells by the virus [21]. These comprise the molecular hinge region of the E-protein, for infection of *Ae. aegypti* and the FG loop of domain III, a co-receptor binding region [22, 23].

MAMMALIAN CELLULAR RESPONSES TO DENV, WNV, JEV AND ZIKV INFECTION

(i) Dengue Virus (DENV)

There are four serologically distinct strains of DENV (DENV 1-4). Dengue pathogenesis is often explained in terms of antibody enhancement, cytokine release and promotion of both apoptosis and pyroptosis in response to viral NS1 protein [24]. Dengue induces apoptosis in hepatocytes, both *in vitro* and *in vivo* [25-29] and up-regulated IL-8 and RANTES have been observed to accompany apoptosis [30].

The DENV non-structural, NS2, NS3, NS5, capsid (C) and E proteins are all known to trigger the extrinsic apoptotic pathway in a variety of cell types, which include endothelial cells, hepatocytes, or immune cells and these processes involve the pro-inflammatory cytokines [30]. The small membrane (M) protein from all 4 serotypes of DENV, on the other hand, induces apoptosis in hepatocytes and neurons [31]. HMGB1 has also been identified as being released during non-apoptotic cell death [32] which, outside the cell, acts as a cytokine [33] and has recently also been identified in the tissue of severe (fatal) dengue victims [34].

Autophagy has been observed in epithelial cells, involving the non-structural viral proteins, NS4 [35], which viruses can exploit to boost energy production by the host cell to assist viral replication [36,37]. DENV induces autophagy and protects against other stress agents [35,36] and this also serves as a means of protection against apoptosis [38]. DENV can induce autophagy by several routes, involving endoplasmic reticulum (ER) stress and altered signaling followed by production of reactive oxygen species (ROS) [39]. The inhibition of ER stress signals limits the ability of DENV to induce autophagy, while increased autophagy ultimately protects from apoptosis and increases the potential of DENV to replicate.

(ii) West Nile Virus (WNV)

While the strategy adopted by WNV appears to be less versatile than that used by DENV, being unable to affect autophagy [40,41], WNV is nevertheless capable of initiating apoptosis in the central nervous system causing neuropathology, or necrosis when the viral load is high [42]. West Nile Virus activates Toll-like receptor 3 and increases the permeability of the blood brain barrier through TNF-α [43]. Distinct strains of WNV affect pathways of ER stress to enable the viral load to be increased. The capsid protein interacts with p53 *in vivo* [44] to activate the intrinsic apoptotic pathway and blocks apoptosis via PI31-Akt [45], while the NS3 viral protein is involved in extrinsic pathway activation in neuroblastoma and cervical cancer cells. Inflammation follows activation of the CASP-9 apoptotic pathway [46] and cell death through the CASP-3 pathway contributes to pathogenesis [47]. Furthermore, WNV has the ability to influence microRNA [48], a capacity which may also extend to other *flaviviridae*. WNV induces ER

stress, to degrade ATF6 (one of the major unfolded protein response pathways whereby the ER responds to a high load of viral proteins by upregulating protein folding machinery) rapidly, causing phosphorylation of a second unfolded protein response pathway) and CHOP-dependent premature cell death. The latter has been proposed as a potential host defence mechanism that is capable of limiting viral replication and which explains neuronal loss in WNV [49].

(iii) Japanese Encephalitis Virus (JEV)

Neutrophil chemotaxis can be induced by JEV [50], which has the ability to influence both the intrinsic and extrinsic apoptotic pathways. The expression of two chemokines, Cxc11 and Cxc12, rapidly increases macrophage numbers and attracts polymorphonucleate leukocytes, key players in the innate immune response. These also serve, during early stages of infection by WNV, as reservoirs of infection, later contributing to clearance [51]. Oxidative stress and programmed cell death can both be induced by JEV infection [52,53]. In neural cells, JEV induces apoptosis by stimulating an up-stream stress response. The NS3 viral protein of JEV, in contrast to both DENV and WNV, induces apoptosis via the intrinsic pathway and JEV can also replicate in the absence of CASP-3, to induce CASP-8 and CASP-9 through a mitochondrion-dependent pathway [54,55]. Inhibition of CASP, however, does not block viral production and JEV appears to depend on mitochondrial apoptosis [56,57] for pathogenesis. In addition, like WNV, JEV activates CHOP [58] and also employs autophagy [59,60] to mediate the pro-inflammatory cytokine response in neural cells.

(iv) Zika Virus (ZIKV)

In general, cell death triggered by viruses, can be either immune-mediated or induced via cell autonomous injury once viral infection has altered the expression of pro- and anti-apoptotic proteins. A number of studies have reported apoptosis as a mechanism of cell death in ZIKV-infected NPCs [61] [62]. However, apoptosis has also been found in adjacent fetal neural cells without evidence of infection and a similar phenomenon of bystander apoptosis has been reported for human immunodeficiency virus [63], DENV [64] and WNV [65]. The proposed mechanism of bystander apoptosis still remains to be elucidated, however, it is likely that cytotoxic factors released by infected NPCs might damage uninfected NPCs [66]. Furthermore, other non-neural cells such as astrocytes, microglia and lymphocytes could become activated in the brain and release pro-inflammatory cytokines that damage uninfected neurons. The complete picture of the immune response to ZIKV is rather complex and warrants further investigation. Similarly to DENV, however, ZIKV can induce autophagy that, during infection of foetal NPCs, can lead to defective neurogenesis [67]. In addition to caspase-dependent cell death, caspase independent cell death has also been reported to cause cell death in infected cells such as epithelial cells, primary skin fibroblasts and astrocytes [68] *in vitro*. This mode of cell death was explained by extensive vacuolization of the ER, which is the major intracellular site of ZIKV replication [69]. The accumulation of ZIKV vacuoles triggers cell collapse defined as paraptosis, involving cytoplasmic vacuolization, independent of caspase involvement [68].

Infection is clearly a complex and multi-faceted event but, one class of cell surface molecules that are present in some form on both mammalian and insect cells and which are coming under increasing scrutiny, are the GAG polysaccharide components of proteoglycans (PGs).

PROTEOGLYCAN ROLES IN HOST-VIRUS INTERACTIONS

The role of PGs, particularly HSPGs is well-documented as a means of attachment of flaviviruses, including DENV [70-73], YFV [74], JEV [75] and TBE [76-78]. The principal PGs are the four integral membrane syndecans and the six glycosylphosphatidylinositol anchored glypicans. In the syndecans, the HS chains are carried on the ectodomain, tending to maintain them distal from the cell membrane whereas, in the glypicans, the HS chains tend to be adjacent to the plasma membrane. There are also many other proteoglycans, carrying GAG chains, including so called 'part-time' PGs, such as CD-44. The proteoglycans bind and regulate a multitude of proteins in the extracellular matrix (ECM) to control key biological processes including cell proliferation and differentiation, wound repair, host defence mechanisms, as well as responses to stress and inflammation. Other more subtle roles are also now emerging for PGs in viral infection. Recently, it was observed that the addition of exogenous heparin (a GAG polysaccharide with close structural similarity to endogenous HS) to cells infected with ZIKV showed a surprising increase in survival and continued viral replication, shown by high viral load in surviving cells, while control cells had died [79]. While the mechanism behind this phenomenon and the extent to which it reflects *in vivo* physiology remains unknown, the possibility that heparin may play a role as a survival factor in trophoblasts has been raised [80], since it was found to influence several anti-apoptotic pathways, including CASP-3. Furthermore, HSPG has been documented as stimulating TNF-α in murine microglia [81] but, the action of heparin seems unlikely to be through binding to TNF-α [82], which is a master regulator of the inflammation response. It is not known currently whether heparin is reinforcing a mechanism that the virus exploits, in order to prolong cell survival and promote viral persistence, or whether it acts through an unrelated mechanism.

A dependency on the HS biosynthetic machinery, in particular on the NDST1 and EXT1 enzymes, has been found using a functional genomics approach for ZIKV infection of HeLa cells [83]. Viral infection results in activation of Toll-like receptor signaling pathways of the innate immune response, sequestration of growth factors, chemokines and cytokines, altered leucocyte adhesion and also influence the behaviour of metalloproteases [84]. The influence of flaviviruses on PG regulation and expression, or the interplay between them, which may exert considerable influence on disease progression, has yet to be explored in detail. Nevertheless, it is noteworthy that the PG, syndecan, is shed in response to stress or damage [85] and, in the case of the bacterium, *Pseudomonas aeruginosa*, is exploited to enhance microbial virulence [86,87]. In addition to the role played by HS in DENV attachment, the NS1 viral glycoprotein that is secreted by infected cells and has been implicated in the disease process, attaches to cell membranes via HS and CS [88]. Thus, GAG components of PGs are involved in several stages of the viral infection process. It will be intriguing to discover the extent to which similar events occur with other flaviviruses. Mechanical stress, which could, under certain strongly inflammatory conditions be relevant to infection, has also been shown to alter PG expression in endothelial cells. The HSPGs contained different polysaccharide (HS) components and exhibited distinct biochemical properties [89]. Stress induced by NO alters the turnover of matrix components [90] which, although recorded for rheumatoid arthritis, may also apply more widely. Interestingly, in the case of infection of endothelial cells by another class of virus, herpes virus, both HSPGs and CSPGs were down-regulated [91].

CONCLUSION

There are several routes available for cell death resulting in varied degrees of longevity and stress on host cells, hence, a variety of mechanisms available for exploitation by the virus. It seems highly likely that the response of cells varies between the causes of the stress to the cells [92] and between different

virus types, the viral load, as well as the cell type and its stage in the cell cycle [93]. The response to inflammation can also be complex, as is the case in kidney where some PGs such as agrin and glomerular basement membrane-associated HS levels are decreased, but CD44 is increased [94]. While there are broad similarities between flaviviruses in this regard, particularly between WNV and JEV, which can both induce neural symptoms, it is not yet clear in mechanistic terms, where this propensity originates. A role for GAGs beyond attachment, which itself could explain some facets of tissue tropism of the flavivruses, in the infection process and cellular responses, is also beginning to emerge. Comparisons between phylogenetically close viruses, such as DENV and ZIKV, will be particularly interesting in this regard. One observation that can be made is that there is little information available concerning PG expression in relevant insect vectors and still less relating the effects of flavivirus infection to PG expression in insects. This represents a significant gap in our knowledge of the life-cycle and infection process of these important viruses.

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Figure Legends

Figure 1. Main cell death modality. Apoptosis is induced by two independent and connected pathways. The intrinsic pathway provokes mytocondrial stress and cytochromeC activation followed by a cascade of caspase activation that terminates with the activation of CASP3, the executor of apoptosis. The extrinsic pathway is triggered by ligation of death receptors that ultimately also activates CASP3. Independently of caspase activation is authophagy that can be triggered by death receptor ligation and

involves lysosome degradation. Pyroptosis is an inflammasome modality of cell death that is mediated by activation of CASP1 and release of the proinflammatory cytokines, IL-1b and IL-18.

Figure 2. The structure of the flavivirus genome. The protein is cleaved co- and post-translationally to form; capsid (C), membrane (M) and envelope (E) structural proteins and also non-structural proteins (NS) -1, 2A, 2B, 3, 4A, 4B and 5.

Figure 1

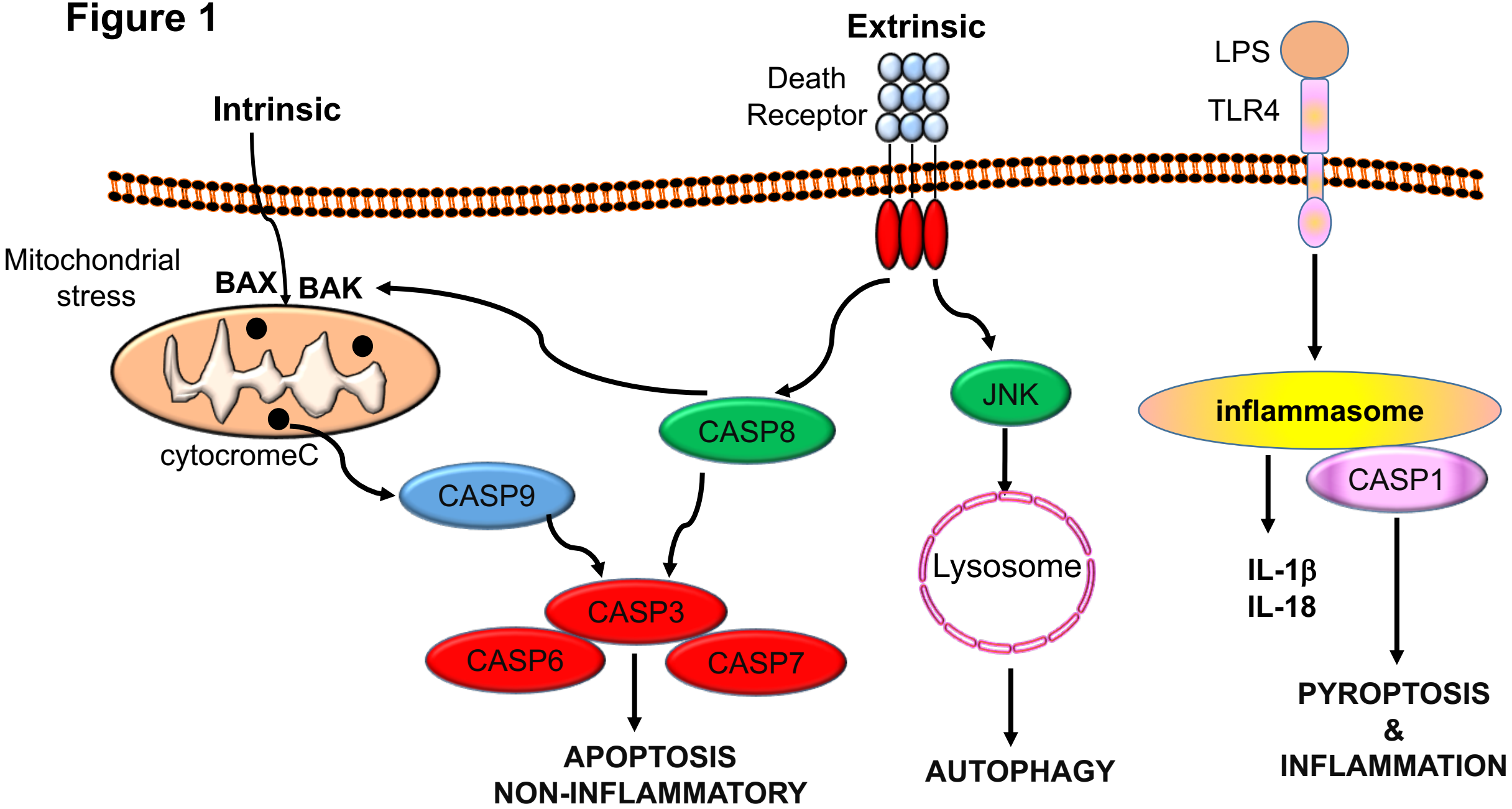
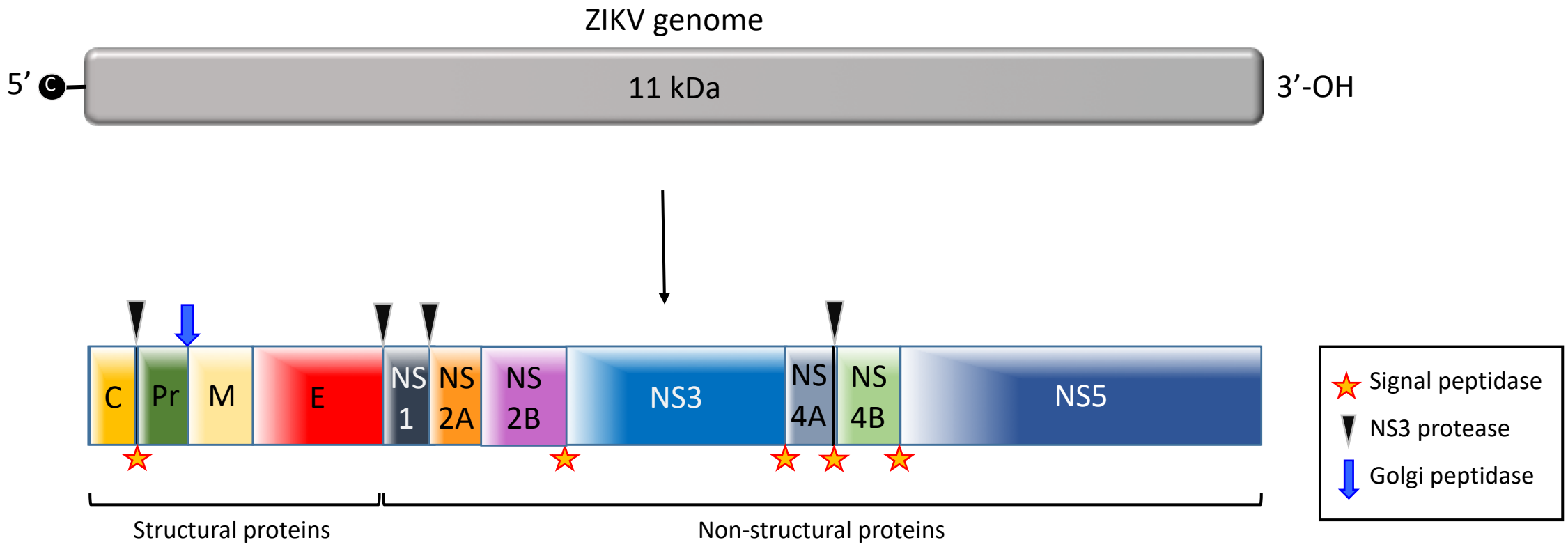

Extrinsic
Intrinsic
Death
Receptor
LPS
TLR4
Mitochondrial
stress
BAX
BAK
cytocromeC
CASP8
CASP9
CASP3
CASP6
CASP7
JNK
Lysosome
inflammasome
CASP1
IL-1β
IL-18
APOPTOSIS
NON-INFLAMMATORY
AUTOPHAGY
PYROPTOSIS
&
INFLAMMATION

Figure 2


ZIKV genome
5’
11 kDa
3’-OH
C
Pr
M
E
NS 1
NS 2A
NS 2B
NS3
NS 4A
NS 4B
NS5
Structural proteins
Non-structural proteins
Signal peptidase
NS3 protease
Golgi peptidase