Review Paper

Importance of NS5 protein to Zika virus survival

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Abstract: ZIKV is the latest addition to an ever-growing list of arboviruses that are causing outbreaks with serious consequences. 14 mild cases were recorded between 1960 and 1980 until the first major outbreak was recorded in 2007 on Yap island followed by more severe outbreaks in French Polynesia (2013) and Brazil (2015) leading to a 20-fold increase in GBS and Microcephaly cases respectively. Various transmission methods have been recorded ranging from *Aedes* mosquito vector transmission to sexual and vertical transmission. No current vaccines or treatments are available but recent studies have taken interest in the NS5 protein which has both the RdRp & MTase domains making it important for viral replication alongside other important functions such as inhibiting the innate immune system thus ensuring virus survival and replication. Structural studies can help design inhibitors while biochemical studies can help understand the various mechanisms utilized by NS5 thus counteracting them can inhibit or abolish the viral infection. Drug repurposing has proven to be an effective tool since hundreds of thousands of compounds can be screened in-silico thus saving time and resources while also having information available on such compounds especially if they are already used to treat other ailments.

Keywords: Zika; NS5; Flavivirus; Arbovirus; TBK1; STAT2; IFN1; IFN3; RdRp; MTase

1. Introduction:

Zika virus (ZIKV) is a single stranded positive RNA arbovirus belonging to the *Flaviviradea* family alongside Dengue, Yellow fever, and West Nile viruses [1]. The 11kb RNA strand has a single open reading frame (ORF) that is flanked by a 5' & 3' untranslated regions that assist in translation (refer to figure 1). The ORF codes a single polyprotein that is processed and cut to yield 3 structural proteins: C→ Capsid, prM→ Pre-membrane, E→ Envelope and 7 non-structural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B & NS5 [2,3].

ZIKV was first isolated from Rhesus monkey 766 in the Zika forest in Uganda in 1947 as part of a Yellow fever surveillance study, thus giving the name MR766 to the African strain. This was followed by the isolation of ZIKV from *Aedes africanus* mosquitoes in the same region a year later [4]. Faye et al. came to the conclusion that ZIKV was introduced in Uganda between 1892 & 1943 and started spreading to neighbouring countries as early as 1935 before its isolation in 1947. This can be represented in the form of 3 clusters; 1- Transmission to the Central African Republic & Nigeria in

1935 (Nigerian cluster). 2- Transmission to Coˆte d'Ivoire & Senegal in 1940 (MR766 cluster). 3-Transmission to Malaysia in 1945 (Asian cluster) [5].
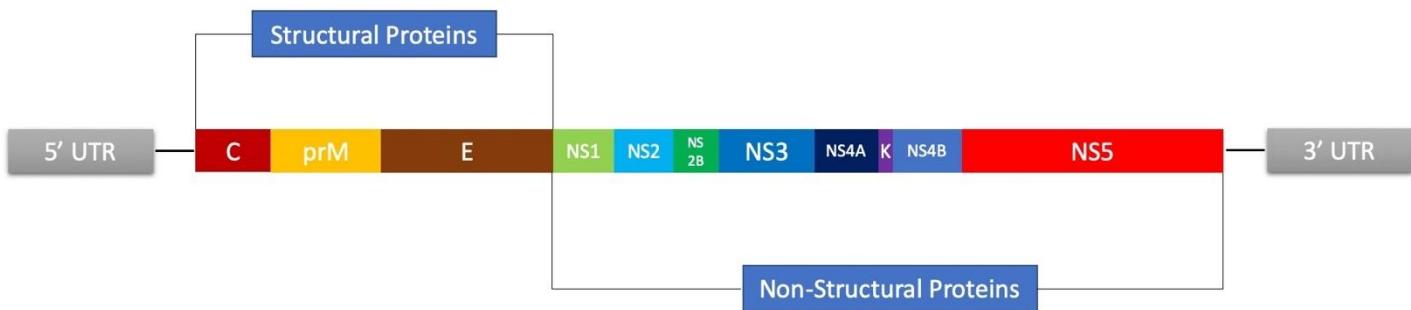
Figure 1. Shows the ZIKV single stranded positive RNA with its single ORF flanked by the 5' and 3' UTR regions. The regions coding for the structural and non-structural proteins are shown as well as the individual proteins.

14 Sporadic cases were reported between the 1960s & 80s without serious implications since the disease presented as a self-limiting febrile illness [6,7]. This changed in 2007 when the first outbreak was recorded on Yap island with 49 confirmed and 59 suspected ZIKV cases. Duffy et al. estimated that approximately 3/4 of the population above 3 years of age showed serological evidence of a recent ZIKV infection [7]. This was followed by an outbreak in French Polynesia in 2013 and finally reaching the Americas by 2015 as predicted [7]. The outbreak in French Polynesia was more severe even though recorded cases only affected 3% of the population since it led to hospitalisation and a 20 fold increase in reported cases of Guillain-Barre syndrome (GBS) [8]. Brazil was a major hub for ZIKV entry into the Americas and suffered heavily by the epidemic since there has been a significant spike in microcephaly and approximately 1.3 million reported cases of autochthonous infection [9]. Finally, the outbreak reached the USA by 2015 [10] and started declining worldwide by the end of 2016 without any signs of resurgence [11].

2. Transmission:

Just like other arboviruses, ZIKV is mainly transmitted through a bite from an infected mosquito. *A. aegypti* and *A. albopictus* are considered to be the main vectors in vast regions [12] but other species like *A. hensilli* which was suggested to be the vector infecting people on Yap island in 2007 [7] and *A. polynesiensis* was suspected to have contributed to spreading ZIKV in the French Polynesia outbreak alongside other local species [13]. ZIKV is mostly found in sylvatic life cycles in which viral reservoirs would usually be established in non-human primates in forests with the occasional human infection via a mosquito bite [14].

Non vector transmission has been documented in many cases including sexual transmission due to the high viral loads in Sertoli cells thus shedding the virus in semen [15,16]. Also, mother to foetus transmission during pregnancy was seen in Brazil thus leading to microcephaly and other developmental issues. Also, high viral loads and RNA were detected in various foatel tissues including the brain, liver, eyes, etc. alongside calcification [9,17]. Furthermore, transmission routes might include the exchange of other bodily fluids like saliva or blood transfusions to name a few [18]. Finally, once a mosquito is infected, the virus follows a similar cycle to that of Dengue virus growing in its gut for 7-14 days before invading the salivary glands ready to infect the next victim once the mosquito feeds [19].

3. Innate Immunity:

After a viral infection, the body relies on its innate immune system to hold the infection at bay while the adaptive immune system can launch a more effective response. The first stage involves the recognition of pathogen associated molecular patterns (PAMPs) via the various pathogen recognition receptors (PRR). These involve the Toll-like receptors and the RIG1-like receptor family. Once activated, they recruit and polymerize the mitochondrial anti-viral signalling (MAVS) protein which in turn activates TNF receptor associated factors (TRAF's) 2, 3 and 6. This is followed by the activation of TANK-binding Kinase 1 (TBK1) thus phosphorylating and translocating Interferon regulatory factor 3 (IRF3) to the nucleus to activate the transcription of IFN1 & 3 [20–23].

IFN1 consists of IFN alpha & beta whereas IFN3 is also known as IFN lambda. Both IFN1 & 3 act directly by massing an anti-viral response while stimulating the adaptive immune system to design antibodies targeting the invader. As seen in figure 2, IFN1 & 3 bind to different receptors; IFNAR1 & 2 for IFN alpha & beta whereas IFN3 binds to the IFNLR1 and IL10R2 receptors, each receptor pair forms a heterodimer on the cell surface. Once activated, both receptor pairs follow a similar downstream signalling cascade as seen again in figure 2. This involves recruiting, phosphorylating and the heterodimerization of JAK1 & TYK2 which leads to the phosphorylation and heterodimerization of STAT1 & STAT2. IRF9 binds to the STAT1/STAT2 heterodimer forming the ISGF3 complex which migrates to the nucleus and binds to various Interferon Stimulated Response Elements (ISRE's) thus transcribing various proteins that deal with the viral infection [24,25]. Zhou et al. conducted a gene array study to determine if both IFN1 & 3 activated different genes but found that all of IFN3's targets are also activated by IFN1 [26].
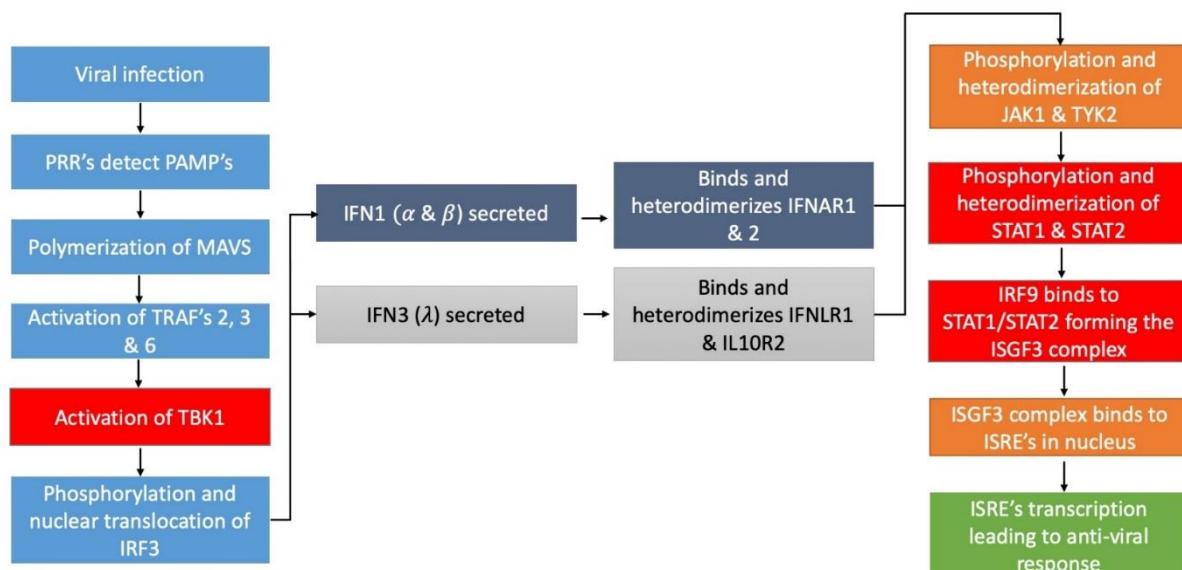
Figure 2. Shows the cascade of events that leads to IFN mediated anti-viral response. The blue pathway leads to the phosphorylation and nuclear translocation of IRF3 thus activating the transcription of the IFN1 & 3 genes. This allows the secretion of both IFN's in which they bind to their respective receptors leading to the activation of the JAK1/TYK2 pathway (depicted in orange) that leads to the formation of the ISGF3 complex thus activating ISRE sites resulting in an effective anti-viral response (depicted in green). ZIKV NS5 interferes at the steps colored in red; NS5 binds to TBK1 preventing its phosphorylation and activation by the TRAF's whereas NS5 binds STAT2 and marks it for proteasomal degradation.

4. NS5:

Protein structure:

The non-structural protein 5 (NS5), is the largest product coded by the ZIKV RNA being around 904 amino-acids long [27,28]. NS5 consists of 2 domains that enhance each other's functions; an RNA-dependent RNA polymerase (RdRp) domain at the C-terminal connected via a linker to a Methyltransferase (MTase) domain at the N-terminal. This makes the NS5 important for viral replication, survival and immune system evasion alongside other roles [29–33]. Zhao et al. found that the ZIKV NS5 protein shares a lot of structural similarities with the Japanese encephalitis virus (JEV) due to conserved residues in the loops and beta sheets forming the MTase domain [33].

Viral Replication:

Potisopon et al. found that both the RdRp and MTase domains interact with one another to increase the efficiency of RNA replication in DENV; once the RdRp domain begins with RNA synthesis, the first stage involves synthesising primers complementary to the 3' end of the genome followed by a conformational change in a transition phase to be able to elongate the RNA. Experiments comparing the speed of each phase between a recombinant NS5 and NS5 polymerase (missing the MTase domain) showed that the MTase domain is required to increase the efficiency of initiation, priming and elongation (6-17 folds higher). A similar outcome was observed in ZIKV NS5 by Zhao et al. and the same can be said for other *Flaviviruses'* NS5 [31,33].

The NS5 MTase domain has been observed by Issur et al. to work alongside NS3 to form the virus' capping mechanism; NS5 is a true guanyly-transferase while the NS3 acts as the RNA triphospahatase (RTase) in the reaction [28]. This is important in preventing the newly formed mRNA from being detected and degraded by the innate immune system while also ensuring its translation by the host translation machinery [34]. The first step involves the hydrolyses of the Adenosine nucleotide in the initiating position at the 5' end of the RNA by the NS3 RTase. Following that, the NS5 MTase forms an enzyme-substrate complex with a GMP molecule which is followed by transferring the GMP to the 5' diphosphate end of the RNA and finally the methylation of the cap at the 2nd OH (2'OMTase activity by NS5) using S-Adenosyl methionine (SAM) as a methyl donor. Furthermore, allosteric enhancement of the NS5 reaction via binding to NS3 at the linker between the RdRp and MTase domains was observed with a maximum increase of 6 times [28].

Host Immune system modulation:

Kumar et al. studied the various effects of ZIKV infection on the innate immune system. There first observation was ZIKV's ability to abolish the host cell's IFN response and signalling; by measuring the levels of *Ifnb* and *Ifit1* using qPCR, they noticed that the peak levels were only attained between 24-48 hours unlike the control's 12 hours. Furthermore, they also detected lower activation levels of the IFIT1 promoter which is both an ISRE and an IRF3 target thus showing that the virus is limiting the innate immune system at both the induction and effector phases. Also, only IFN1 and 3 were affected by ZIKV whereas IFN2 response was unaffected. This can be attributed to ZIKV's NS5 protein binding to and proteasome-dependent degradation of cellular STAT2 within 24 hours while STAT1 was not affected thus explaining the lack of effect on IFN2 signalling [30].

Grant et al. studied this mechanism and found that it slightly differs from the mechanism utilised by DENV in which ZIKV NS5 does not recruit the E3 ubiquitin ligase UBR4. Furthermore, they concluded that ZIKV NS5 stays in the nucleus until cytosol STAT2 levels increase after which the NS5 exits the nucleus to bind to and degrade the STAT2 thus preventing it from forming the

ISGF3 complex and activating ISRE targets. This effect was reversed with the treatment of proteasome inhibitors. In addition to that, NS5 uses its MTase domain to bind to STAT2 preparing it for degradation but requires the full length NS5 to complete the process [30,35]. Finally, ZIKV NS5 only interacts with human and other non-human primate STAT2 whereas wild type mice are immune whereas IFN deficient mice were susceptible to ZIKV infection thus showing the importance of this signalling pathway in fighting infection [35,36].

Lin et al. suggested a mechanism by which ZIKV NS5 abolishes the IFN response via an upstream pathway; inhibiting the effect of IRF3 thus preventing the transcription of IFN1 & 3 genes (refer to figure 2). This is attained through preventing the phosphorylation and nuclear translocation of IRF3, i.e. IRF3 levels remained constant after infection unlike the 70% decrease in phosphorylated IRF3. ZIKV NS5 is able to do this by binding to TBK1 via its ULD domain, both the MTase and RdRp domains are required as the absence of one showed no decrease in phosphorylated IRF3. Furthermore, TBK1 binds to the groove formed by the MTase/RdRp linker and forming this complex might interfere with TRAF 6's interaction and binding with the TBK1 SDD domain due to their close proximity. Finally, this leads to the inability to activate TBK1 which in turn cannot phosphorylate, and activate IRF3 thus abolishing IFN1 & 3 transcription [37].

Targetting NS5 for anti-viral treatments:

As seen above, the NS5 protein has various important roles in ensuring the virus' replication and survival thus making it an interesting candidate for anti-viral targeting. Any effects changing the way the RdRp or MTase domains behave can lead to disastrous outcomes for the virus; folding in a different way thus preventing interactions with other ZIKV and host proteins while reducing or abolishing the enzymatic activity of either domains would also prove fatal to the virus. One can argue that other proteins such as the structural E protein, NS1, NS3 and NS4A also inhibit the induction and signalling pathways involving IFN1. Different groups found this to be true, but Kumar et al. proved that NS5 is more effective at doing so since it inhibited the activation of the IFIT1 promotor by 70% compared to 40% via the E and NS4A proteins [30,37].

Inhibition of the NS5 RdRp and MTase domains can be exploited to distort or abolish the domains' ability to carry out its functions. This can be done through either competitive or allosteric inhibition. Furthermore, nucleoside analogues like sofosbuvir have been successfully used to treat previous viral infections such as hepatitis C by the early termination of viral mRNA synthesis and has shown inhibition towards ZIKV NS5 [38,39]. Another interesting venue is repurposing previous drugs in which hundreds of thousands of drugs are screened against the structure of ZIKV NS5 to determine any inhibitory effects, this might include anti-virals, anti-biotics, anti-cancer and anti-parasitics [40,41]. This can help save time and resources since we already have information about the current drugs and their safety.

Multiple articles can be found reviewing the current developments of ZIKV NS5 inhibition [42–45], but no drugs have been approved yet even though some look promising. Clinical trials need to be conducted to test the safety and efficacy of these drugs before mass treatment.

5. Conclusion:

In summary, ZIKV outbreaks are not active at the moment but there are various hotspots where the virus is endemic and there are no guarantees that there will be no more future epidemics thus an effective vaccine or treatment needs to be developed. The NS5 protein has shown its importance in virus survival and replication alongside various interactions with both host and viral proteins. This makes it an interesting target since disrupting it can lead to a cascade of events proving disastrous for viral survival. Finally, once an effective treatment is developed, it can be repurposed to treat other Flaviviruses as well due to the high similarity and conservation of the NS5 protein.

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