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Molecular Mechanism of Interaction between Influenza Virus and Host Cells: An Attempt to Understand Signaling and Cellular Pathways

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Abstract

Influenza virus infection is highly contagious and difficult to treat, because of the emergence of different strains formed via antigenic variations. Influenza is a causative agent of acute viral respiratory infection with a broad infectious range that includes all age groups and is linked with high mortality during pandemics, epidemics, and sporadic outbreaks. Influenza A virus is generally known to be limited to the respiratory system however it can also lead to systemic complications such as myocarditis, encephalitis and death. Additionally, already known vaccines induce only temporary immunity and no worldwide vaccine is available. Thus, there is a need to study the detailed mechanisms and interactions of the virus within the host, which would help to discover specific target-based treatment. This review is focused on the different signal transduction pathways which are responsible in eliciting an immune response against the influenza virus infection. Viral entry, endocytosis, Toll-like receptor pathway, Retinoic acid-inducible gene pathway, Jak-Stat pathway, Nucleotide-binding oligomerization domain-like receptor pathway, Apoptosis, Cell cycle interference, m-RNA surveillance pathway, RNA transport, MAPK signaling pathway, virus budding, and release were studied in detail to understand the interaction between the virus and the host. More light has been put upon the intricate pathways that take place within the host and how specific viral proteins regulate those pathways to promote the viral growth.

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Introduction

Influenza A virus is a causative agent for acute respiratory disease marked by high fever, weakness, cough, acute rhinitis, headache and by inflammation of the upper respiratory tract and trachea. The infection is at peak during winters and affects people of all age-groups, but in particular, children, neonates, the elderly as well as the co-morbid population. The high-risk population suffer from complications like cardiac diseases, diabetes, hemorrhagic bronchitis and pneumonia. Each pandemic and interpandemic slightly differ with respect to pathological changes [1]. Influenza transmission is mediated by direct contact, coughing, sneezing and through aerosols as droplets more than 5 micron in size. The highly pathogenic subtypes are H5 and H7 causing infection in chicken as well as other birds leading to the classical avian plague, called bird flu [2]. Influenza viruses predominantly showcases high morbidity and mortality rates.

The outbreaks caused by the virus originally started in the Mid seventeenth century, since then various pandemics occurred during 1700, 1889, 1918, 1957 and 1968. The three major pandemics that took place in the past century included Spanish Flu which took place in 1918 (H1N1), Asian influenza of 1957 (H2N2), and Hong Kong Flu that occurred in 1968 (H2N3) resulting in 40 million deaths, 1-2 million deaths and 0.75-1 million deaths respectively. The outbreak of classical avian influenza was observed in Netherlands and Belgium in spring 2003 by influenza A H7N7 and H5N1 epidemic in South-East Asia in the year 1998 which severely affected the human population [3]. The Northern hemisphere experiences influenza outbreaks between October and March whereas the Southern hemisphere experiences them in April and August. The influenza epidemics result in approximately 500,000 deaths annually worldwide [3]. Among the mentioned, the deadliest pandemic occurred in 1918 with a death rate of 675,000 in the US, making upto 50 million of the worldwide population, followed by the 1957 pandemic responsible for the death rate of 66,000 in the US. According to the latest WHO data published in 2018, influenza and pneumonia deaths in India reached 616,531 or 6.99% of total deaths. The mortality rate with respect to age is 63.11 per 100,000 of population which ranks India 54th in the world [99]. A mixed influenza infection caused by A/H3N2 and A/H1N1 subtypes has been observed since 1977. Although these subtypes only became predominant and known in 2005.

Influenza A is an enveloped virus with a segmented, single-stranded, negative-sense RNA genome with a lipid-bilayer membrane belonging to the Orthomyxoviridae family. It is a causative agent of acute respiratory disease, Influenza in humans. Hemagglutinin (HA), Neuraminidase (NA) and M2 are the integral proteins followed by Matrix protein (M1) found below the lipid membrane, polymerase basic protein 1 and 2 (PB1), (PB2) and Non-structural proteins (NS1) and Nuclear export protein (NS2) are the proteins found in influenza A virus [4]. The family Orthomyxoviridae comprises of Influenza virus A, Influenza virus B, Influenza virus C and Thogotovirus genera out of which only Influenza virus A & Influenza virus B poses a threat to public health. Influenza virus A further consists of subtypes that are characterized by their surface glycoproteins, HA and NA. 16 Subtypes of HA(H1-16) and 9 subtypes of NA (1-9) have been isolated till now. In addition to them, two subtypes have been isolated from bats which are HA(H17 and H18) and NA(NA10-NA11) [100]. Segmented genome and error-prone RNA dependant RNA polymerase of influenza A are responsible for antigenic drift and antigenic shift leading to disruption of Host adaptive immune response. The error-prone RNA dependant RNA polymerase of the Influenza virus is subjected to high levels of mutation which tend to help in the invasion of the host immune system. In addition, due to the lack of proofreading, the segmented genome results in genetic re-assortment between two different influenza viruses leading to co-infection of the same host. Thus, these are the major reasons for phenomena like antigenic drift and antigenic shift [5].

Viral entry

The viral entry is initiated by recognition of the Sialic acid receptor (also known as N -acetylneuraminic acid) present on the host cell surface which is followed by receptor binding and entry of the virus leading to infection. The common host cell receptor for Influenza A virus is sialic acid that is covalently linked at the end of oligosaccharides on glycoproteins or glycolipids and is found on surface of various cell types and cell secreted products. The probability of infections is magnified due to sialylation which masks cells, microorganisms and their products from being recognized by the host immune system [6]. Each HA subunit possesses the binding site for sialic acid receptor and hence, influenza virus exhibits a multivalent binding to the host cell surface. HA of influenza subtypes infecting humans prefer a sialic acid having α 2,6-glycosidic linkage to glycoproteins and glycolipids whereas the HA of avian influenza subtypes prefer α 2,3 glycosidic linkage [7]. The α 2,6-linkages are predominant in human alveolar and bronchial epithelial cells, alveolar macrophages, Type II pneumocytes and plasmacytoid dendritic cells whereas α-2,3-linkages are found in abundance on avian respiratory epithelial surfaces [8]. Figure 1 represents the overview of the influenza virus life cycle inside the host cell.

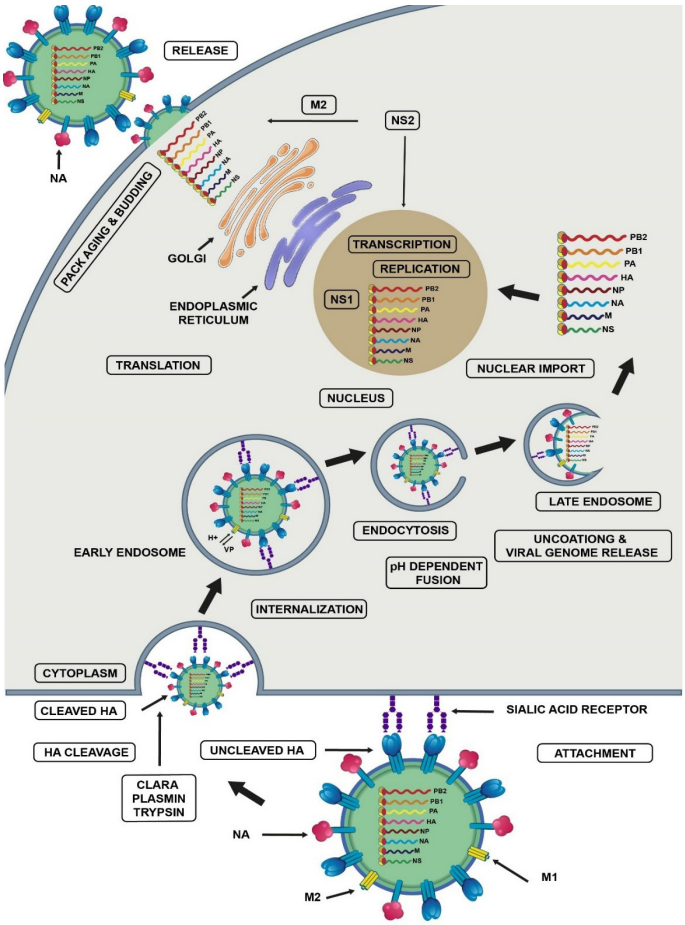

Figure 1: The overview of Influenza A virus lifecycle inside the host cell.

Human airway trypsin-like protease (HAT) and Transmembrane protease serine member 1 and 2(TMPRSS2) are proteases belonging to the family of type II transmembrane serine proteases. They contain a short N-terminal cytoplasmic domain, a variable stem region, a transmembrane sequence and a C-terminal catalytic serine protease domain [9]. HAT and TMPRSS2 present in the human airways are responsible for proteolytic activation of influenza A virus [10,11]. Since HA is synthesized in the form of a precursor requiring cleavage, newly synthesized HA is cleaved by HAT before or during the release of progeny virions and HA of incoming viruses before endocytosis at the cell surface, while TMPRSS2 mediates cleavage of newly synthesized HA within the cell and is unable to assist the proteolytic activation of HA of incoming virions. HA possessing a monobasic cleavage site is cleaved by TMPRSS2 in the Golgi apparatus while assembly or by HAT on the plasma membrane either during attachment and entry into the cell or during budding of virions [12]. After viral attachment, the acidic pH of the cytoplasm induces endocytosis forming an endosome that engulfs the entering viral particles. The endosome is then transported to the nucleus. Final step is marked by the fusion of the viral membrane with the host endosomal membrane and is facilitated by HA. This results in the release of viral genome into the nucleus. Further, viral transcription and replication takes place in the nucleus. The host proteases cleave glycosylated HA into HA1 and HA2 which gives rise to a fusion peptide at the N terminus of HA2. The encapsulated viral particles are translocated near nucleus in endosomes and are further subjected to membrane fusion due to the acidic conditions of late endosome. This fusion results in release of viral RNA-dependent RNA polymerase-associated nucleocapsid (RNP) into the nucleus where viral transcription begins. Influenza virus can perform clathrin- independent endocytosis as well as clathrin-dependent endocytosis [13]. The viral particles get encapsulated into coated vesicles which are halted at the plasma membrane and suggest that an active actin framework leads and translocate these vesicles to microtubules where early endosomes are located. The protein sorting system of the host cell is essential for movement and direction of early endosomes to late endosomes along microtubules [14].

In the late endosome, HA facilitates fusion of endosomal membrane with the viral membrane at low pH (5.0). At the acidic pH, HA experiences a conformational change in order to introduce the fusion peptide into the host cell membrane. Further, conformational change of HA is constrained and the HA1 trimer is stabilized by sialic acid binding [15]. Polyvalent interactions take place between the receptor molecules present on the endosomal membrane and trimeric HA while the viral particles are enclosed in the endosome during endocytosis. Here, the fusion peptide located at the N terminus of HA2 is liberated due to low pH before the refolding of HA. Hence, preventing binding of HA to sialic acid on the endosomal membrane is necessary to permit HA2 refolding and emphasizes on importance of lower pH [16]. A step involving dissociation of HA from binding to sialic acid on the endosome membrane may be necessary for complete refolding of HA2. The fusion peptide maintains the metastable conformation of HA trimer and contributes to low pH triggering of HA2 refolding. Low pH causes conformational changes in the Matrix protein (M1) necessary for accomplishing membrane fusion and release of Viral ribonucleoprotein (RNP). In addition to this, it isolates M1 protein from RNP to expose the mechanism underlying nuclear import [17].

During influenza viral entry, the cleavage of HA precursor that is HA0 into HA1 and HA2 is followed by formation of the fusion peptide. The polybasic residues present before the cleavage site permit various host proteases (such as clara, plasmin and trypsin) to bring about cleavage, enhancing the virulence of the virus. The hydrogen bond interaction provided by the side chains of ionizable residues with main chain amides of the fusion protein stabilizes it in the pocket [18]. On the other hand, conformational changes in the vicinity of the pocket leads to release of the fusion peptide from the pocket in highly pathogenic strains. This liberated fusion peptide can penetrate the host target membrane when it is elevated by the refolded HA. Initially, the fusion peptide catalyses the hemi-fusion by blending the outer section of the two lipid bilayers and further forms a fusion pore where both the sections are merged. The fusion peptide then forms an α-helix and enters the lipid bilayer. The fusion peptide helix formed in the lipid bilayer comprises of a twist that separates the framework into two parts, as shown in the figure 2a.

With a pH of 5.0, introduction of the firmly folded helical fusion peptide causes membrane distress in order to mediate lipid interactions between juxtaposed membranes. However, at neutral pH the entry of the fusion protein into the lipid bilayer is reversible. Meanwhile, entry of the fusion peptide at lower pH results in irreversible membrane fusion in order to give rise to an active fusion pore. The fusion peptide endures a step where it diffuses in the lipid bilayer to achieve self-associated status preceding the hemi-fusion step of the lipid bilayer. This may be a rate-limiting step and would seek contribution of multiple copies of HA [19]. Enlargement of the initial fusion pore results in complete fusion of the two membranes causing release of the contents in the viral particle. The participation of the transmembrane domain in HA is necessary in this last step. The transmembrane carries out packaging and anchoring of HA in the envelope of the viral membrane. Additionally, it contributes in the last step of membrane fusion [20]. If HA is attached to a cell membrane via GI-linked lipids, it may initiate hemi-fusion with other cell membranes, however it will fail to complete the fusion. This indicates that only a full-length HA along with the transmembrane domain can facilitate membrane fusion. The expansion of pore at lower pH and release of viral contents are necessary during the final stage [21].

a) A protein-catalysed membrane fusion process occurs in case of enveloped viruses like influenza A whereas non-enveloped viruses lyse the endosomal membrane or form a pore [22]. Clathrin-mediated endocytosis takes place by accumulation of receptors and bound ligands into clathrin-coated pits (CCPs), which detaches from the plasma membrane forming clathrin-coated vesicles (CCVs). Further, uncoating and fusion of CCVs with other endocytic vesicles occurs [23]. The following steps are included in influenza viral infection of cells:Internalization of virus through receptor-mediated endocytosis;

b) Trafficking of the internalized virus from early to late endosomes at low pH;

c) HA mediated viral membrane and endosomal membrane fusion at acidic pH;

d) Transportation of viral RNPs into the nucleus for viral replication and transcription of viral genes [24].

Upon exposure to low pH (~5), influenza viruses fuse with their endosomal compartments, allowing their genome to be

released into the cytoplasm to initiate infection [22]. It takes 3 minutes for CCP formation on an average once the virus binds to the host cell. The clathrin signal lasts for only about a minute, after which the clathrin coat quickly dismantles. As soon as the uncoating of the vesicles takes place, the virus is subjected to microtubule dependent transport [25]. Influenza viruses undergo at least one acidification step before their entry into early endosomes (to pH ~6). The endocytic compartments are monitored by Ras-associated binding proteins (Rab) and other factors. Initially in early endosomes the internalized components are organized towards distinct fates and the recycled ligands and receptors are brought back to the cell surface, whereas material meant to be degraded is transported to lysosomes [26]. Rab5x proteins collaborate with early endosomes and control early endocytic traffic. These endosomes are regulated by Rab11 and possibly by Rab4. Hence, late endosomes were found to associate with Rab7 and Rab9 proteins [22]. Three-stage transport behaviour prior to fusion is observed in which the virus initially moves slowly in the vicinity of the cell (stage I), then further it implements a rapid and unidirectional movement towards the nucleus (stage II), followed by an irregular, often bi-directional movement in the perinuclear region (stage III) [27]. Studies show that stage I movement is actin-dependent, whereas stage II movement is engaged by dynein on microtubules, and stage III includes both minus- and plus-end-directed movements on microtubules [27]. While stage II and III signify the movement of virus-containing vesicles or endosomes within the cell, stage I partially occurs on the cell surface [22]. The acidification step occurring prior to virus fusion signifies the necessity for an additional acidification step (probably to late endosomal pH) to achieve fusion [25]. NA eliminates local sialic acids and releases the ineffective HA associations. Avian subtypes possess HAs exhibiting enhanced specificity for receptors containing α-2,3-linked sialic acids having a “linear” presentation while HA from influenza A virus human subtypes prefer an α-2,6 linkage, which leads to a “bent” presentation [28].

The endocytosis may occur in a clathrin-dependent manner, including dynamin and the adaptor protein Epsin-1, or by micropinocytosis [29]. When the virus is translocated to the endosome, its low pH leads to conformational change in HA exposing the fusion peptide via activation of M2 ion channel. Entrance of the M2 ion channel facilitates acidification within the viral particle, liberating the packaged vRNPs from M1, which allows the trafficking of the vRNPs to the host cytoplasm subsequent to HA-mediated fusion [4]. The viral-endosomal membrane fusion is a multistage process. The cleavage of HA by host cell proteases into two subunits, HA1 and HA2 is necessary for the same. In addition to this, cleavage of HA facilitates exposure of fusion protein located on the N-terminus of the HA2 due to endosomal pH change [30]. After the exposure, the fusion peptide enters into the endosomal membrane, while the C-terminal transmembrane domain (TMD) attaches HA2 in the viral membrane, forming a pre-hairpin structure. The HA2 trimers refold forming a hairpin that commences the positioning of the two membranes in vicinity of each other. The hairpin assembles then further breaks down into a six-helix bundle, while doing so, the two membranes come in close proximity facilitating the construction of the lipid stalk, and the resulting fusion of the two membranes [4]. As explained in the figure 2b.

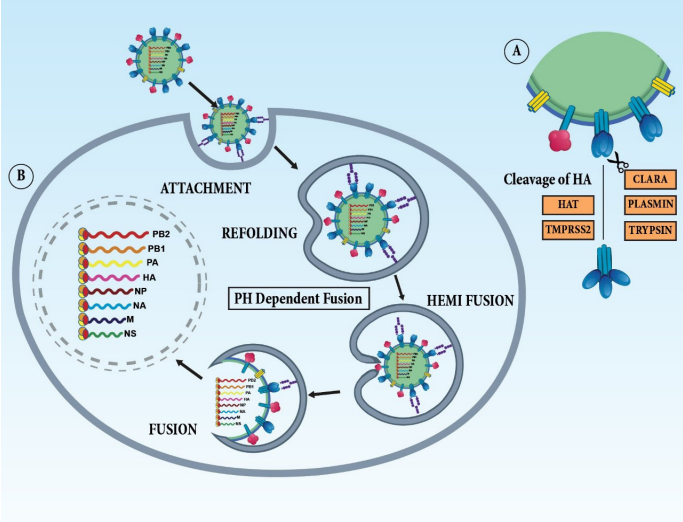

Figure 2: Endocytosis of Influenza A virus. A] Cleavage of Haemagglutinin (HA) via proteinases clara, plasmin and trypsin and proteolytic activation of Influenza A virus by HAT (human airway trypsin-like protease) and TMPRSS2 (transmembrane protease serin member 1 and 2(TMPRSS2). B]Steps involved in the process of endocytosis namely attachment,refolding,pH dependent fusion, and release of the viral genome into the nucleus.

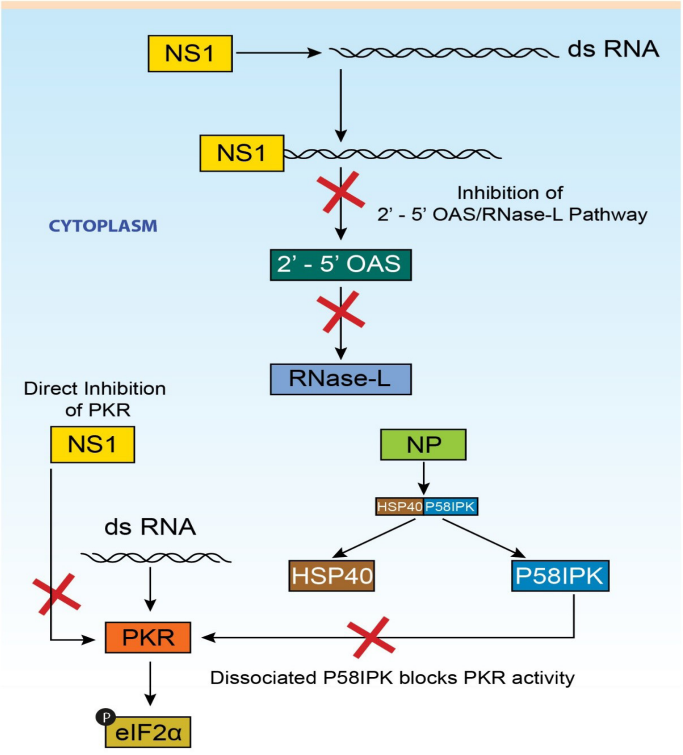

Figure 3: Late endosomal pathway. A] NS1 protein binding to the dsRNA resulting in the inhibition of RNase-L pathway. B] Dissociation of P58IPK from the HSP40-P58IPK complex regulating the PKR activity. C] NS1 protein inhibiting the PKR activity directly.

Protein kinase C (PKCβII) which is required for endocytic trafficking of influenza A viral particles gets activated when HA protein binds the cell receptor. In absence of PKCβII activity, the viral particles accumulate in cytoplasmic vesicles which are known as late endosomes. Therefore, PKCβII activity results in late endosomal sorting event. NS1 protein of influenza A virus binds to target dsRNA and inhibits the function of cellular proteins like polyadenylation specificity factor (CPSF) and poly(A)-binding protein II (PABII) which are necessary for cellular pre mRNA processing. Further NS1 is employed in the inhibition of

IFN-α/β–induced 2'-5'-oligo A synthetase /RNase L pathway by seizing dsRNA away from 2'-5'-oligo synthetase [31]. Nucleoprotein (NP) interacts with human chaperone heat shock protein 40 (HSP40) thereby regulating Protein kinase R (PKR) activity since PKR influences the innate immune response in mammalian cells. Under normal conditions HSP40 is present in a complex with P58IPK which is a 58 kilo Dalton cellular inhibitor in an inactive state. During Influenza infection P58IPK dissociates from HSP40 and blocks the PKR activity. Thus, in mammalian cells the NP of influenza A virus results in decreased phosphorylation of PKR and its substrate that is *eIF2α and releases P58IPK from HSP40- P58IPK complex. However the NS1 protein also plays a role in direct inhibition of the PKR activity [32].* As shown in figure 3.

Toll like receptor (TLR) signaling pathway

Toll-like receptors (TLRs) are essential for recognition of pathogen-associated molecular patterns (PAMP) in the innate immune system, triggering downstream signaling pathways resulting in the induction of innate immune responses by production of inflammatory cytokines [33]. TLRs signaling pathway helps in employing specific adaptor molecules, resulting in the activation of the transcription factors like NF-κB and Interferon Regulatory Factor [IRFs], which influence the outcome of innate immune responses [33]. Interferon Regulatory Factors play important role in immune response as IRF3, IRF5, and IRF7 carry out production of type I interferons downstream of pathogen recognition receptors (PRRs) that are responsible for detection of viral RNA and DNA whereas IRF9 is involved in interferon-driven gene expression.

A TLR comprises of an ectodomain containing leucine-rich repeats (LRRs) that mediate PAMPs recognition, a transmembrane domain, and a cytoplasmic Toll/IL-1 receptor (TIR) domain that brings about initiation of downstream signaling. The ectodomain exhibits a horseshoe-like structure, and interaction of TLRs with their respective PAMPs or Damage Associated Molecular Patterns (DAMPs) form a homo- or heterodimer along with a co-receptor or accessory molecule [36]. Followed by PAMPs and DAMPs recognition, TLRs employs TIR domain-containing adaptor proteins including Myeloid differentiation primary response protein (MyD88) and Toll/Interleukin-1 Receptor-domain-containing adapter-inducing interferon-β (TRIF) that are responsible for initiating signal transduction pathways that leads in the activation of NF-κB, IRFs, or Mitogen-activated protein (MAP) kinases which further regulate the expression of cytokines, chemokines, and type I IFNs that provides protection to the host from microbial or viral infections [33]. Intracellular localization of TLRs is necessary for ligand recognition as well as for blocking TLRs from coming into contact with self-nucleic acids, which could lead to autoimmunity [34]. Nucleic acid-sensing TLRs undergo proteolytic cleavage in the endosome by cathepsins B, S, L, H, and K and asparginyl endopeptidase to achieve a functional form that carries out ligand recognition and begins signalling [35].

MyD88, TRIF, TIRAP/ MyD88 adaptor-like (MAL), or TRAM members of a set of TIR domain-containing adaptors are differentially recruited by individual TLRs. MyD88 assists all TLRs in activation of NF-κB and MAPKs for the initiation of inflammatory cytokine genes [33]. MyD88 is recruited to cell surface TLRs such as TLR2 and TLR4 by a sorting adaptor called TIRAP. Along with TRIF, TLR3 and TLR4 promote an alternative pathway resulting in the activation of IRF3, MAPKs for induction of type I IFN, NF-κB and inflammatory cytokine genes. Meanwhile, TRAM is selectively assigned to TLR4 but not TLR3 to link between TRIF and TLR4. TLR3 directly interacts with TRIF, however this interaction requires phosphorylation of the two tyrosine residues located in the cytoplasmic domain of TLR3 using the Epidermal growth factor (ErbB1) and Bruton tyrosine kinase (Btk) [36,37]. Therefore, depending on the usage of the adaptor, TLR signaling is widely differentiated into two pathways - MyD88-dependent and TRIF-dependent pathways.

Myddosome is a complex formed by MyD88 with family members of Insulin Receptor Kinase (IRK). Activation of interleukin-1 receptor-associated kinase 1 (IRAK1) by IRAK4 takes place during Myddosome formation which is then auto-phosphorylated at various sites and released from MyD88 [38]. IRAK1 forms an association with the RING-domain E3 ubiquitin ligase TRAF6. TRAF6 with the help of Ubiquitin-conjugating enzyme UBC13 and UEV1A, results in K63-linked polyubiquitination of both TRAF6 and the Transforming growth factor β-activated kinase 1 (TAK1) protein kinase complex [39]. TAK1 belongs to the MAPKKK family and forms a complex with the regulatory subunits Transforming growth factor β-activated kinase binding proteins - TAB1, TAB2, and TAB3, which further interact with polyubiquitin chains formed by TRAF6 leading to TAK1 activation [40]. Further, two different pathways are activated by TAK1 resulting in activation of the IKK complex-NF-κB pathway and -MAPK pathway. The IκB kinase (IKK) complex consist of the catalytic subunits IKKα and IKKβ and the regulatory subunit NEMO (NF-κB essential modulator, also called IKKγ). TAK1 attaches to the IKK complex via ubiquitin chains, allowing it to phosphorylate and activate IKKβ. The IKK complex brings about phosphorylation of the NF-κB inhibitory protein IκBα, which is subjected to proteasome breakdown, allowing translocation of NF-κB into the nucleus in order to initiate proinflammatory gene expression [33]. Activation of MAPK family members including as Extracellular Regulated Kinase (ERK1/2), p38 and c-Jun-N-terminal Kinase (JNK) responsible for activation of AP-1 family transcription factors or stabilization of mRNA for regulation of inflammatory responses is also resulted from TAK1 activation [41]. This pathway is initiated with interaction of TRIF with TRAF6 and TRAF3. TRAF6 recruits the Receptor interacting protein-1 (RIP-1), which in turn interferes with TAK1 complex and activates it, ensuing the activation of NF-κB and MAPKs and induction of inflammatory cytokines. Meanwhile, TRAF3 employs the IKK-related kinases TBK1 and IKKi with NEMO for phosphorylating IRF3. Eventually, IRF3 forms a dimer and enters the nucleus from the cytoplasm, where it gives rise to the expression of type I IFN genes [42,41]. The E3 ubiquitin ligases belonging to the Pellino family are involved in TLR signaling. TBK1/IKKi phosphorylates Pellino-1 and trigger ubiquitination of RIP-1, suggesting that Pellino-1 faciliates TRIF-dependent NF-κB activation by employing RIP-1. Pellino-1 also regulates IRF3 activation by interacting with DEAF-1, a transcription factor that facilitates attachment of IRF3 to the IFNβ promoter. As shown in figure 4a [43]. An inositol lipid known as Phosphatidylinositol 5-phosphate (PtdIns5P) is said to regulate IRF3 activation as interacts with both IRF3 and Traf family member-associated NF-κB binding Kinase 1(TBK1), and promotes complex formation between TBK1 and IRF3. The availability of TBK1 to IRF3 facilitated by PtdIns5P leads to phosphorylation of IRF3 in a convenient manner. During virus infection, production of ptdins5p is carried out by a kinase known as Phosphoinositide Kinase, FYVE-type Zinc-finger containing (Pikfyve) [44].

Activation of MyD88-dependent and TRIF-dependent pathways is carried out by TLR4 and regulated by various molecules

in order to initiate immune responses. The regulation of these pathways is crucial for balanced production of Type I interferons and other inflammatory cytokines for avoiding autoimmune conditions as well as tumorous cell growth [33]. Along with the MyD88 complex, TRAF3 is also incorporated into the TRIF complex during TLR4 signaling where TRAF3 within the MyD88 complex is then disintegrated resulting in TAK1 activation. Therefore, TRAF3 along with encouraging TRIF-dependent pathway activation it also inhibits the MyD88- dependent pathway. The ubiquitination of MyD88 and TBK1 is brought about by an E3 ubiquitin ligase known as Neuregulin receptor degradation protein-1 (NRDP-1) which promotes the breakdown of MyD88 leading to reduced production of inflammatory cytokine. It also enhances the activation of TBK1 which favours the type I IFN production [45]. MHC class II molecules residing in endosomes of antigen-presenting cells interact with the tyrosine kinase Btk through the costimulatory molecule CD40 and sustain Btk activation. The activated Btk interacts with MyD88 and TRIF to facilitate the activation of the MyD88-dependent and TRIF-dependent pathways resulting in increased production of inflammatory cytokines and type I IFNs, respectively [46].

Plasmacytoid DCs (PDCs) are a subcategory of DCs having ability to secrete large amounts of type I IFN during viral infection [41]. In PDCs, TLR7 and TLR9 function as primary sensors for RNA and DNA viruses, respectively. Remarkably, the production of type I IFN by PDCs depends on a complex containing MyD88, IRF7, TRAF3, TRAF6, IRAK4, IRAK1, IKKα, OPNi, and Dock2 [47]. IRF7 is phosphorylated by IRAK1 and/or IKKα in this complex and further transported into the nucleus to control the expression of type I IFN as shown in figure 4b. This MyD88-IRAK4-TRAF6 complex initiates NF-κB-dependent inflammatory cytokine induction. Viperin is responsible for formation of MyD88-IRAK1-TRAF6-IRF7 signaling complex in the lipid bodies which further activates IRAK1 via lysine 63- linked ubiquitination [48]. First, TLR9 enters Vesicle Associated Membrane Protein 3 (VAMP3)-positive early endosomes after CpG-DNA stimulation, promoting MyD88-IRAK4-TRAF6-dependent NF-κB activation. TLR9 further moves to Lysosome-associated membrane protein 2 (LAMP2)-positive lysosome-related organelles (LROs), where it integrates TRAF3 in order to activate IRF7 and induce type I IFN [33]. Adaptor protein 3 (AP3) attaches to TLR9 and regulates the movement of TLR9 to LROs, and is necessary for type I IFN induction [34]. Nevertheless, AP3 is not necessarily required for TLR9-dependent type I IFN induction stimulated by DNA-antibody immune complexes (ICs) in PDCs. The intracellular compartment facilitating type I IFN induction using DNA-antibody ICs is regulated by the autophagy pathway. CD14, a glycophosphatidylinositol-anchored protein, initiates immunoreceptor tyrosine-based activation motif (ITAM)-mediated Spleen Associated Tyrosine Kinase (Syk) and phospholipase C gamma 2 (PLCγ2)-dependent endocytosis to facilitate the entry of TLR4 into endosomes in order to activate the TRIF-dependent signaling. It also acts as a co-receptor along with TLR4 and Myeloid Differentiation factor 2 (MD-2) for recognition of lipopolysaccharide. CD14 also mediates TLR7 and TLR9-dependent induction of proinflammatory cytokines [49].

The negative regulators of TLR signaling pathway avert the unnecessary or elevated immune responses leading to inflammatory diseases or autoimmunity. The negative regulators suppressing the activation of the MyD88-dependent pathway are ST2825, Suppressor of cytokine signaling 1 (SOCS1), and Casitas B-lineage Lymphoma proto-oncogene b (Cbl-b) , whereas activation of the TRIF-dependent pathway is suppressed using Sterile alpha and armadillo motif-containing protein (SARM) and TRAM adaptor with GOLD domain (TAG). (Verstak) [101]. In addition to this, these molecules cooperate with MyD88 or TRIF to inhibit them from interacting with the TLRs. TRAF6 and TRAF3 activation is negatively regulated by A20, Ubiquitin-specific protease 4 (USP4), cylindromatosis gene (CYLD), TRAF family member-associated NF-kappa-B activator (TANK), TRIM38, small heterodimer partner nuclear receptor gene (SHP), SOCS3 and Deubiquitinating enzyme A (DUBA), respectively [50]. Meanwhile, TAK1 activation is hindered by TRIM30α and A20. Also, the transcription factor NFκB is negatively regulated by B cell lymphoma 3 protein (Bcl-3), NF-kappa-B inhibitor delta (NFKBID), Nuclear receptor related 1 (Nurr1), Activating Transcription Factor 3 (ATF3), and PDLIM2, while IRF3 activation suppressed by Peptidylprolyl Cis/Trans Isomerase, NIMA-Interacting 1 (Pin1) and RAUL [51].

The NS1 protein of type A influenza viruses plays an active role in overpowering the innate immune system signaling pathways activated by the recognition of PAMPs by PRRs like TLR3, TLR4, RIG-I, and MDA5 system. The suppression of the immune response should also provide an efficient replication of infected virus in respiratory epithelial. The signaling pathways facilitate defence by inhibition of IRF-3 phosphorylation, leading to repressed induction of IFN-α/β, and IFN-λ1, 2, and 3 [52]. Furthermore, hinderance in activation of NF-κB and AP-1 in cells infected by influenza virus result in decreased proinflammatory cytokine production such as IL-8 and TNF-α [53]. NS1 protein blocks the type I IFN induction, the IFN-induced antiviral activity, the binding and segregation of dsRNA, the intrusion with the host mRNA processing, the enablement of favoured viral mRNA translation and the inhibition of DC activation. As shown in figure 4c [54].

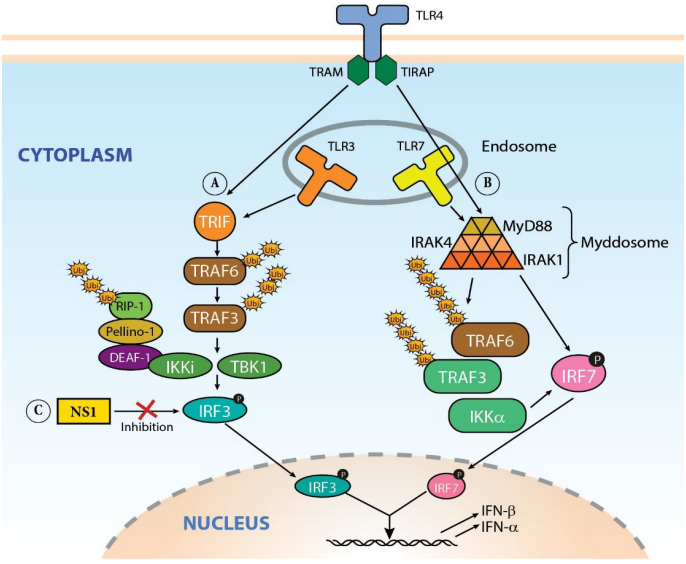

Figure 4: Toll-like receptor signaling pathway. A] TRIF-dependent pathway. TLR3 activates TRIF which interacts with TRAF6 and TRAF3. Ubiquitinated TRAF6 and TRAF3 further employ IKKi and TBK1 for phosphorylation of IRF3 which facilitates IFN α expression. B] MYD88-dependent pathway. TLR7 interacts with the myddosome complex involving IRAK1, IRAK4 and MYD88 protein family. This complex further gets associated with TRAF6, TRAF3 and is also responsible for the phosphorylation of IRF7 which after its interaction with IKK α translocates to the nucleus and expresses IFN β.C] NS1 Inhibition. NS1 protein inhibits IRF3 phosphorylation leading to suppression of interferon induction and hindering the NF-κB pathway.

RIG like signaling pathway

RIG like Receptors (RLRs) are a type of intracellular pattern recognition receptor involved in the recognition of viral particles by the innate immune system. RLRs include of retinoic acid-inducible gene I (RIG-I), laboratory of Genetics and Physiology 2 (LGP2) and melanoma differentiation-associated gene 5 (MDA5). Their RNase helix domain also known as DEX/DH box promotes RNA binding [58]. The RLRs signaling pathway leads to transcription factor activation which further triggers type 1 interferon (IFN) production and antiviral gene expression that induces an intracellular immune response against viral infection. The RLRs in PRRs are a class of RNA helicases located in the cytoplasm, recognizing non-self-viral RNA by attaching their PAMPs to their RNA ligands resulting in the production of type I interferons and the production of inflammatory factors by influencing the activation of RLRs and downstream signaling molecules [55]. The transduction of downstream signals is triggered due to conformational changes of RNA which in turn is altered via ATP hydrolysis.

RIG-I and MDA5 both possess two N-terminal CARD domains (caspase activation and recruitment domains) which allow them to interact with other molecules containing this domain. These interactions result in binding of RIG-I/MDA5 to an important linker molecule called MAVS (mitochondrial antiviral signaling protein). It is also known as IPS-1/VISA/Cardif, which also comprises the CARD domain, triggers activation of factor IRF-3, IRF-7 (interferon regulatory factors) and transport NF-κB into nucleus, subsequently leading to the activation of several antiviral genes like interferon, interferon-stimulated genes, and pro-inflammatory factors thus, inhibiting the replication and spread of the virus. The RIG-I and LGP2 consist of a repressor domain (RD domain) or C-terminal domain, encouraging inactivation of RIG-I and LGP2 in the absence of a viral infection [56].

RNA consisting of a 5'-end triphosphate (5'ppp) tail is necessary for recognition by RIG-I, since it assists RIG-I to differentiate between viral RNA and host cell RNA. The key to distinguish is that host cell RNA comprises of a cap structure at the 5' end whereas a 5'ppp structure is absent in tRNA and rRNA. Meanwhile complete elimination of 5'ppp prohibits activation of the RIG-I signaling pathway entirely. Recognition of the viral RNA by RLR is followed by activation of mitochondrial antiviral signaling protein (MAVS), also known as IFN-β promoter stimulator I (*IPS-1*). Once the RLR identifies the viral RNA, it triggers and transduces the signal to the TRAF3, TBK1 kinase and IKK-I complex, further phosphorylating and activating IRF3/7. This activated IRF3/7 is transferred to the nucleus. Thus, leading to the production of type I interferon [55].

Activated IPS-1 has a potential to pass TRAF2/6 (tumour necrosis factor (TNF) R-associated factor 2/6) or FADD (Fas-associated death domain), RIP1 (receptor interacting protein-1), and TRADD (Tumour necrosis factor receptor type 1-associated DEATH domain protein), with Caspase 8 /10 pathways emitting signals to IKK complexes (IKKα, IKKβ, IKKγ), eventually resulting in phosphorylation of NF-κB and IκBα complexes. The Phosphorylated IκBα disintegrates from NF-κB and activated NF-κB enters the nucleus to facilitate the production of pro-inflammatory factors and inflammatory chemokines. Studies suggest that RIG-1 can be regulated by ubiquitin-protein ligase (E3). TRIM25 (tripartite motif containing 25) collaborates with RIG-I as a ubiquitin ligase leading to the ubiquitination of K17 lysine residues in the CARD domain and this ubiquitination results in activation of RIG-I and IPS1. Also, E2 ubiquitin-conjugating enzyme 5 contributes in the activation of RIG-I signaling pathway, which may be involved in K63 ubiquitination of IKKγ, located downstream of MAVS, and triggers the activation of TBK1 and IRF/NF-κB by IKKγ. Moreover, the RIG-I pathway can also be negatively regulated via ubiquitination as the ubiquitin ligase E3, Ring finger protein 125 (RNF125) attaches the K48 ubiquitin chain to RIG-I and MDA5, facilitating their degradation by the proteasome [57].

The three RLRs play an important role in eliciting innate defences within myeloid cells, epithelial cells, and cells of the central nervous system. IFN exposure enhances RLR expression while it is maintained at low levels in resting cells. Further, MDA5 expression being virus inducible in cells that lack the IFN receptor, indicates that RLR expression can be determined by a direct virus-inducible signal [55]. RIG-I and MDA5 recognize variety of viruses and mediate the production of IFN and initiation of an antiviral response. They also exhibit a few structural similarities and are organized into three distinct domains: i) an N-terminal region comprising of tandem caspase activation and recruitment domains (CARD), ii) a central DExD/H box RNA helicase domain possessing the capacity to hydrolyze ATP and to interact and unwind RNA, and iii) a C-terminal repressor domain (RD) placed within the C-terminal domain (CTD) if the RIG-I is involved in autoregulation. Meanwhile similarly organized, LGP2 does not possess the N-terminal CARDs and is believed to facilitate regulation of RIG-I and MDA5 signaling [58].

IFN expression is signalled by RIG-I in response to poly-uridine motifs that contain interspersed C nucleotides. The RNA motifs rich in poly-uridine help to enhance RLR signaling to ssRNA PAMPs. All components including, PAMP RNA ligand composition, along with 5'ppp are important factors of a non-self-signature for RIG-I recognition. Thus, 5'ppp along with the secondary motifs such as poly-uridine runs, and specific short dsRNA structures jointly help to detect a viral RNA as a non-self component. Furthermore, RNA cleavage products generated by the 2',5'-linked oligoadenylate-activated RNase L ribonuclease can trigger RIG-I- and MDA5-dependent IFN production, and this may lead to the magnification of antiviral response via production of RLR substrates [55].

RIG-I and MDA5 signaling enables production of IFN in response to a viral infection by an adaptor protein known as IPS-1. IPS-1 is a CARD comprising, membrane-linked protein which is vital for RLR-dependent IFN production in response to a viral infection [57]. Signaling initiates by the detection of viral RNA PAMPs which causes activation of RLR and by using homotypic CARD-CARD interactions reacts with IPS-1. In addition to the N-terminal CARD, analysis of sequence reveals the presence of a transmembrane domain on the IPS-1 C-terminus that acts as an anchor to intracellular membranes. Studies have positioned IPS-1 on the outer membranes of the mitochondria, the peroxisomes membranes and mitochondria-associated membranes [56]. As shown in figure 5, IPS-1 is interacted by RIG-I and MDA5 which helps to relocate the RLRs to IPS-1-associated membranes where they and downstream signaling molecules collect to form an IPS-1 signalosome that carries out IFN production. Signal transduction ends with the activation of a transcription cascade leading to production of IFN and achievement of antiviral state [55].

Crucial transcription factors which are involved in RLR signaling and the IPS-1 signalosome comprise mainly of interferon regulatory factor 3 (IRF3), IRF7 and NF-κB. IRF3 and IRF7 transcription factors are initially present in a dormant form that

are further phosphorylated due signal transduction by non-canonical IκB kinases IKKε or TBK1. Homo and heterodimers are formed by phosphorylated IRF3 and IRF7 that accumulate in the nuclei where target sequence binding occurs which leads to gene transcription. In contrary, activation of NF-κB requires the IKK complex mediated. Inhibitory subunit IκBα is phosphorylated which is then exposed to ubiquitin-dependent degradation caused by proteasomes. Activated forms of IRF3 and/or IRF7 and NF-κB together with the ATF-2 and c-Jun transcription complex and the transcription enhancer CBP-p300 accumulate as part of an enhanceosome to dictate IFNβ transcription. In major cell types excluding the PDCs, IRF3 is fundamentally expressed whereas expression of IRF7 is comparatively less, until its induction takes place in existence of IFN in a positive feedback loop. IRF-3 and components of the NF-κB activation cascade have been recognized as componets of an IPS-1 signalosome in numerous studies [59]. Production and secretion of IFNβ which is the result of RLR cascade, binds to the IFN receptor in an autocrine or paracrine way to drive the JAK-STAT signaling and the expression of interferon stimulated genes (ISGs) by ISGF3. This signaling aids to intensify the IFN response by enhancing the expression of IFN-α subtypes in a positive feedback loop. Other ISGs include proteins encoded with direct antiviral activity such as viperin [55].

Apart from inducing the expression and formation of IFN and ISG products, viral infection and signaling by the RLRs also encourages the expression of the IFN-λ, family of IL-10-related cytokines collectively known as type III interferon (IFN-λ) and various proinflammatory cytokines to control infection. RLR signaling by RIG-I, TBK1, IPS-1 and IRF-3 are essential for the production of IFN-λ following Newcastle disease viral infection, the promoter regions present upstream IFN-λ genes disclose numerous cis-acting components for IRFs and NF-κB binding. Collectively, type I IFN and IFN-λ induction both occur by analogous pathways, and that RLR signaling also encourages the expression of IFN-λ which is required to control virus infection. In case of the pro-inflammatory response, latest research suggests that RLR signaling facilitates this response by means of two pathways. The first comprises of IPS-1-CARD9-Bcl-10-dependent transcription of pro-inflammatory genes, which comprises mostly of NF-κB target genes. The second includes association of RIG-I with ASC protein to trigger caspase-1-dependent activation of inflammasome and converting pro-inflammatory cytokines such as IL-1β and IL-18 into their developed forms. Thus, RLR signaling may cause divergence beyond or independently of IPS-1 to facilitate the inflammatory response that is linked with production of interferon and adaptive immunity [55].

Signaling of RLR to IRF3, IRF7 and NF-κB is controlled by a complex signaling transduction event that include machineries previously allied with the tumor necrosis factor receptor I (TNFRI) and TLR signaling pathways. IPS-1 networks with the TRADD protein and its employment is important for RLR signaling. TRADD is found to be in a complex with Fas associated death domain-containing protein (FADD), and the death domain kinase RIP1. As a result of signaling by the IPS-1 associated TRADD-FADD-RIP1 complex, there is employment of TANK and NEMO to the IPS-1 signalosome to allow IRF3 and IRF7 activation by IKKi or TBK1, IKKα-IKKβ-dependent activation of NF-κB, and production of IFN [60]. However the connection between these molecules is still not clear, knockdown of SINTBAD or NAP1 impairs virus-induced signaling through both the RLR and TLR pathways. A latest study shows the finding of a novel human IKKε splice variants, that exhibit variability in their capacity to interact with TANK, NAP1 and SINTBAD, and this acts as a foundation for dictating the diverse signaling functions of IKKε within the RLR signaling program [61]. The RLR signaling pathway further interconnects with the inflammasome signaling pathway. NLRX1 (Nod9) belongs to nucleotide-binding domain and leucine-rich-repeat-containing (NLR) family in the regulation of RLR signalling [62].

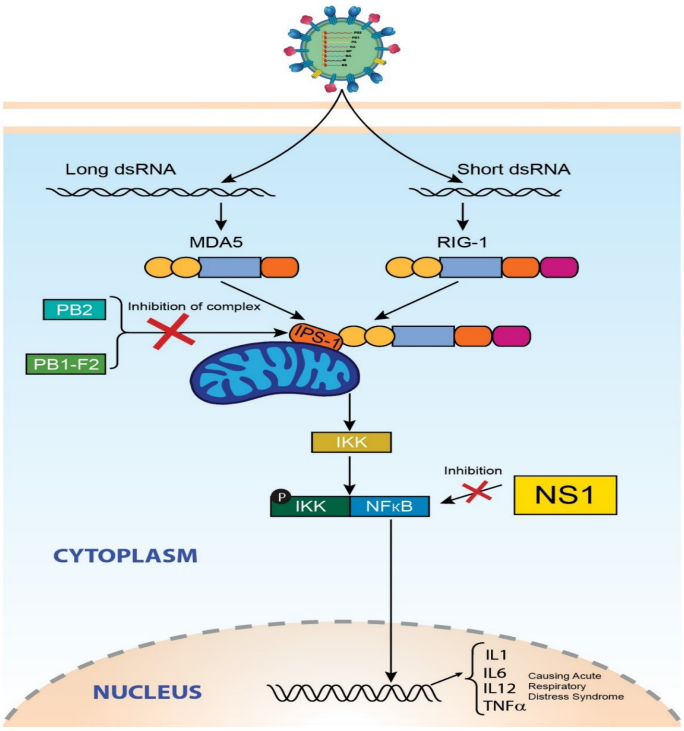

Figure 5: RIG-like receptor signaling pathway. RIG1 and MDA5, members of the RLRs recognise and binds with RNA (DEX/DH domain). This complex then activates the MAVS, downstream IKK complex leading to the phosphorylation of NF- κb and IKb α complexes. Activated NF- κb enters the nucleus and enhances the production of IL1, IL6, IL12 and TNF α causing acute respiratory distress syndrome.

NLRX1 is situated at the outer membrane of the mitochondria, and it interacts with IPS-1 blocking RLR-dependent IFN induction by disturbing the IPS-1 interactions with signaling-active RLRs. Decreased NLRX1 expression increases virus-induced signaling and reduces virus replication, suggesting that NLRX1 acts as a negative regulator of RLR-induced antiviral responses. On the contrary, NLRC5 another member belonging to NOD-like protein family collaborates with RIG-I and MDA5 but not IPS-1 to hinder RLR-mediated IFN responses. NLRC5 interacts with IKKα and IKKβ resulting in inhibition of their phosphorylation and further preventing their NF-κB activating activities [63]. Hence, studies have shown that siRNA silencing of NLRC5 expression leads to enhanced NF-κB transcriptional activity to induce IFN production and signaling of the antiviral response [55]. The antiviral compounds induced due to NF-κB expression include namely IL1, IL6, IL12, TNFα. These released components cause high fever leading to acute respiratory distress syndrome as shown in figure 5. Acute respiratory distress syndrome, or ARDS, which is an inflammatory lung injury occurs due to accumulation of fluids in alveoli in the lungs preventing them from filling up with air and leads to extensive shortage of oxygen levels in the blood also known as hypoxemia. Thus decreasing the optimum oxygen level required for other organs such as brain, heart, kidneys and stomach for them to function [64]. IL-8, MCP1 [(Monocyte Chemoattractant Protein-1

(MCP-1) also called as the CCL2-Chemokine Ligand-2)], RANTES (Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted, also called CCL5- Chemokine ligand 5), IP10 [C-X-C motif chemokine ligand 10 (CXCL10) also known as Interferon gamma-induced protein 10 (IP-10) or small-inducible cytokine B10] and ICAM1 (Intercellular Adhesion Molecule-1, also known as CD54), all of these components play a major role in chemo-attraction of monocytes and macrophages leading to a phenomena known as viral myocarditis.

The janus kinase-signal transducer and activator of transcription (JAK-STAT) Pathway

The JAK-STAT pathway plays a very important role in signal transmission from cell-membrane receptors to the nucleus. It is essential for a large range of cytokines and growth factors leading to vital cellular events, such as lactation, hematopoiesis, and development of the immune system and mammary glands [65]. Janus kinases (JAKs) are a family of PTKs which play an essential role in facilitating signal transduction of numerous cytokines, and even many non-cytokine regulatory molecules. These enzymes contain two potential active sites because of which they are named after Janus, the Roman god with two faces. STAT is a major component of the Jak-stat signaling cascade which is also known as 'signal transducers and activators of transcription'. As the name proposes, these proteins structure a fundamental piece of cytoplasmic signal transduction started by certain regulatory molecules and activate transcription of specific genes [66].

In presence of a viral infection such as influenza A, IFNα (interferon alfa), IFNβ (interferon beta) and IFNγ (interferon gamma) are released which activates the jak-stat pathway. The released interferon α and β bind to the IFNAR (interferon alfa/beta receptor) and interferon γ binds to IFNGR (interferon gamma receptor). This later in turn phosphorylates IFNAR1-bound tyrosine kinase 2 (TYK2) which then further leads to phosphorylation of IFNAR2-bound Janus kinase 1 (JAK1). The receptor-bound kinases phosphorylate STAT1 and STAT2 respectively at amino acid positions 701 and 690. The phosphorylated STAT1 and STAT2 get dimerized by shared SH2-phosphotyrosine interactions. Phosphorylated STAT1–STAT2 heterodimer later gets dissociated from their respective receptors and IRF9 is appointed which leads to the formation of complex in cytoplasm known as ISGF3. ISGF3 will be transported into the nucleus and attaches to the promoter region of interferon-stimulated response element (ISRE) to activate the transcription of ISGs (interferon-stimulated gene) [67].

These genes lead to the production of several factors such as MxA (The human myxovirus resistance protein) which is a chief mediator of the interferon-induced antiviral response towards a large variety of viruses. MxA can assemble itself into highly ordered oligomers and can form a ring-like structure around liposomes, which causes liposome tubulation. Thus, oligomeric rings around tubular nucleocapsid structures may be formed by MxA, thus hampering their transcriptional and replicative functions [68]. The oligoadenylate synthase (OAS) proteins are double-stranded RNA-activated enzymes which are induced by IFNs. Exogenous nucleic acids are detected by these proteins and antiviral pathways are executed against them [69]. ADAR gene encodes the enzyme responsible for RNA editing by site-specific deamination of adenosines. It destabilizes double-stranded RNA by changing adenosine to inosine [70]. PML gene acts by offering instructions for a protein that acts as a tumor suppressor, which means it prevents cells from growing and dividing too quickly or in an uncontrolled way which is basis of malignancy [71].

The IFN-γ receptor on the other hand recruits JAK1 and JAK2 to phosphorylate STAT1. STAT1 homodimers, a.k.a. gamma interferon-activated factor (GAF), translocate to the nucleus and stimulates ISG expression by binding to gamma interferon-activated sites (GAS) [72] which further leads to the activation of CIITA (Class II trans-activator) a transcriptional coactivator that is a master regulator of major histocompatibility complex (MHC) gene expression. It encourages *de novo* transcription of MHC class II genes and enhances constitutive MHC class I gene expression [73].

Another important set of proteins are called SOCS proteins (Suppressor of cytokine signaling proteins) contribute in a simple negative feedback loop in the JAK/STAT cascade in which the activated STATs stimulate transcription of the SOCS genes and the resulting SOCS proteins bind to phosphorylated JAKs and their receptors leading to termination of the pathway [72]. Influenza A viruses interrupts JAK/STAT signaling partially by reducing the expression of the IFN receptor. NS1 aims at both chains of the IFNAR receptor by suppressing their expression at the transcriptional level. In addition, (as shown in figure 6) NS1 induces the upregulation of SOCS3 which causes an increased negative regulatory effect towards the pathway, thus shutting it down; However, this response by influenza virus effects the immune response by the JAK STAT pathway in a negative way [74].

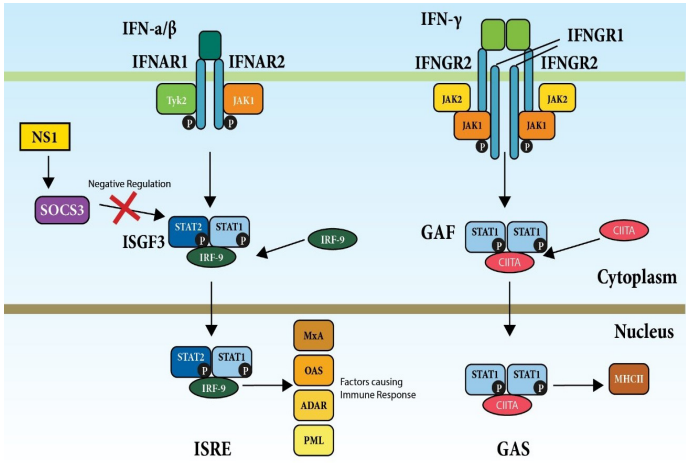

Figure 6: JAT-STAT PATHWAY. Presence of NS1 leads to the upregulation of SOCS3 which acts as negative regulator and thus shuts down the pathway, due to which the Stat complex cannot produce the factors required for generation of an immune response.

Nucleotide-Binding oligomerization domain (NOD)-like receptors signaling pathway

NOD-like receptors (NLRs), are sensors present within the cell directed towards PAMPs that enter the cell by phagocytosis or pores and cell stress associated DAMPs. The Nod-like receptors are recognition receptors present in the cytoplasm that precisely detect non-self-components such as pathogenic microorganisms and other hazardous components in different parts of the organism by interacting with TLRs, thus controlling the immune response. NLRs act as critical activators of innate immune responses resulting in cell damage and infections which may lead to the expression and further activation of stress kinases, inflammatory caspases and interferon response factors (IRFs).

NLR mainly consist of three different domains: firstly, the leucine rich repeat (LRR) in the C-terminus, which plays a significant role in the ligand recognition. Secondly, the N-terminal effector domain such as CARD (caspase activating and recruitment domain) and PYD (Pyrin domain), are linked to NLR receptor molecules, downstream adaptor proteins and effector molecules. Thirdly, there is occurrence of the NACHT (neuronal apoptosis inhibitory protein cIITA HET-E TP1) domain in the middle, which plays a very important role in NLR oligomerization and activation. The human NLR is divided into many components to name a few such as NOD (nucleotide-binding oligomerization domain), NALP (neuronal apoptosis LRR pyrin-domain protein), CIITA (class II transactivator), NAlP (neuronal apoptosis inhibitory protein) and IPAF (ICE-protease-activating factor). Among which, NOD and NALP are the chief members. One of the first discovered NLR is NOD. It has been found that humans have more than 20 NLR members, most of which are widely expressed, such as NOD1, which is largely expressed in adult tissue cells, and NOD2 is seen to be expressed in bone marrow-derived cells, particularly immune cells such as macrophages and neutrophils.

NOD1 and NOD2 are cytosolic proteins that sense intracellular bacterial peptidoglycan and stimulate signal transduction by NF-κB and MAPK activation. Self-oligomerization of both NOD1 and NOD2 takes place due to activation by their similar ligands, both undergo a conformational change and through homotypic CARD–CARD interactions they permit the recruitment of the CARD comprising adaptor i.e., Receptor-interacting protein kinase 2 (RIPK2). This leads to the formation of a multi-protein complex involved in signaling named as "Nodosome," which causes inflammatory and anti-microbial responses mediated by NF-κB and MAPK. While NOD1 and NOD2 initiate the formation of Nodosome, other NLRs assemble macromolecular inflammasome complexes [75].

The inflammasome is a multiprotein intracellular complex, which is commonly formed in response to several pathophysiological stimuli. They are localized within the cytosol, despite which these inflammasome structures are capable enough of initiating an effective immune response against viruses, bacteria and fungi. Structure of an inflammasome includes a sensor (NLR), an adaptor protein [ASC (apoptosis-associated speck-like protein containing CARD)] and an effector molecule (pro-CASP1). ASC is a two-parted protein consisting of a PYD (Pyrin domain) and a caspase recruitment domain (CARD) in resting cells, caspase-1 is present in an inactive pro-form (zymogen) called pro-caspase-1. Caspases are said to be executioners of the apoptotic response, and also play an important role in activation of inflammasome [76]. Most commonly studied inflammasome are the NLRP3 inflammasome. NLRP3 is exposed to a wide array of PAMPs and DAMPs that initiate the assembly and activation of the inflammasome. The NLRP3-inflammasome formation comprises of a two-step process. The first step (or signal 1) involves TLR-NF-κB-driven induction of inflammasome components, as basal expression of NLRP3 in resting cells is not satisfactory for efficient activation of inflammasome. The second activation step (or signal 2) encourages the NLRs to undergo homotypic oligomerization which leads to the assembly of the inflammasome. NLRP3 inflammasomes are brought together in response to foreign RNA both during in vivo and in vitro testing. Extracellular adenosine triphosphate or ATP released from dying or damaged cells also lead to activation of NLRP3-inflammasome by either paracrine or autocrine detection of ATP by the P2X7 purinergic receptor. It is also clear that ATP released from phagocytosed dying cells acts similarly on P2X7 and produces a stimulus leading to opening of pannexin-1 (PANX1) channels, thus resulting in potassium (K^+) efflux and permitting other components to further engage and activate NLRP3 [75].

Further the inflammasome activation initiates through the auto-coactivation of caspase-1, resulting in subsequent cleavage of pro-interleukin-1β (pro-IL-1β) and pro-interleukin-18 (pro-IL-18) into their mature and active forms (IL-1β and IL-18, respectively). Pyroptosis may occur as a result of secretion of these cytokines. Pyroptosis is a term used to describe the inherently inflammatory process of CASP1-dependent programmed cell death. However in case of a viral infection by influenza A virus the NS1 plays a very important role in the inhibition of the NLRP3 inflammasome formation because of which there is repressed maturation of pro-interleukin-1β (pro-IL-1β) and pro-interleukin-18 (pro-IL-18) and no formation of the active forms (IL-1β and IL-18, respectively), thus inhibiting Pyroptosis (as shown in figure 7) [76].

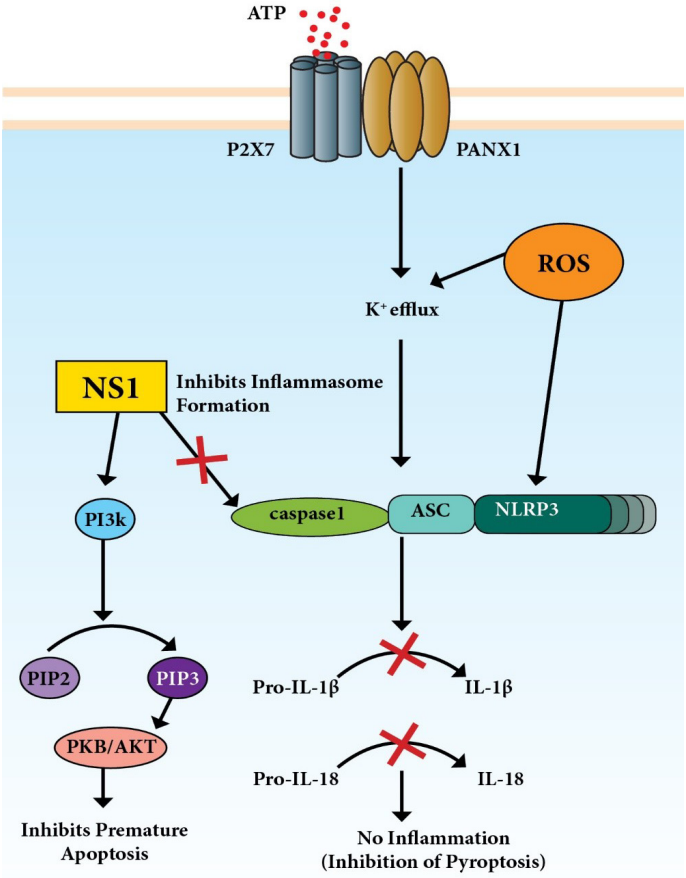

Figure 7: NOD-LIKE RECEPTOR SIGNALING PATHWAY. Inflammasome formation is inhibited because of the viral component NS1, due to which pro-IL-1β and pro-IL-18 do not get activated into their mature and active forms, IL-1β and IL-18 thus not inducing inflammation. NS1 also activates PKB/AKT which inhibits premature apoptosis which favours the virus to continue its infection.

Newly introduced signaling mediators which are induced in presence of influenza virus are phosphatidylinositol 3-kinase (PI3K) and its downstream effector Akt/protein kinase B (PKB). PI3K contains regulatory (p85) subunit and enzymatic (p110) subunits, each present in numerous isoforms. The active form of the enzyme displays a protein kinase as well as a lipid kinase activity. Various cellular processes such as metabolic regulation, cell growth, proliferation, and survival are controlled by the kinase. The NS1 protein of influenza activates PI3K, as a result of this activation, there is formation of phosphatidylinositol 3,4,5-trisphosphate from phosphatidylinositol 4,5-bisphos-

phate which is membrane bound, this acts as a second messenger to employee pleckstrin homology domain-containing proteins, such as Akt which is also known as PKB and phosphoinositide-dependent kinase 1. Akt/PKB is a major PI3K effector and further gets activated by phosphorylation at the Thr308 and Ser473 amino acid residue. This activation however inhibits premature apoptosis (as shown in figure 7) of the infected cell thus allowing the infection to prevail [77].

Apoptosis

Apoptosis, or programmed cell death, is an important cellular signaling response which is generally observed after viral infections. In this process, individual cells undergo controlled self-destruction in response to a diverse range of stimuli [78]. Majorly apoptotic pathways depend on an intracellular cascade comprising the catalytic activation of cysteine-dependent aspartate-directed protease (caspases). Caspases are divided into three major groups namely initiator caspases (caspases 2,8 & 9), effector caspases (caspases 3, 6 and 7) and inflammatory caspases (human caspases 1, 4 & 5; and mouse caspases 1 and 11). Initiator caspases are activated through homophilic dimerization, as a result causing autocatalytic cleavage. The activated form of the initiator caspases will cleave and activate effector caspases. The active form of the effector caspases further activates CAD or caspase-activated DNase which is required for disintegrating the genomic DNA of the cells by terminating inhibitor of CAD (ICAD). During virus infection, several cytokines trigger the activation of initiator caspases. These cytokines are known as death ligands, which are type-II transmembrane proteins that include tumour necrosis factor (TNF), TNF-related apoptosis-inducing ligand (TRAIL) or Fas ligand (FasL). In the naive state, death ligands exist as homophilic trimers, while their specific receptors known as death receptor or DR exist as monomers on cell surfaces. The trimeric death ligand complexes interact with the monomeric DRs which causes accumulation of the receptors and adaptor proteins which further bind to the receptors cytoplasmic tail. Among the adaptor proteins, Fas-associated protein with death domain (FADD) recruit's procaspase-8 to its cytoplasmic tail and leads to the activation of caspase-8. Caspase-8 in the active form cleaves and in turn activates caspase-3. Pro-apoptotic B-cell lymphoma 2 (Bcl2) family proteins, such as BH3 interacting domain death agonist protein (Bid) is also cleaved by Caspase-8 [79]. Bid exists in an inactive form in the cytosol, but due to caspase-8 cleavage, the C terminal (tcBid) is translocated to the mitochondria. The signal for cell death is transmitted by proapoptotic factors such as Bax and Bak that further cause mitochrondrial damage and cytochrome c release. Bax is generally found in the cytoplasm or loosely attached to the mitochondria. In response to the apoptotic stimuli, Bax changes conformation and translocates to the mitochondria where it undergoes oligomerization and becomes an integral membrane protein. In contrast, Bak is often found at the mitochondria and the endoplasmic reticulum (ER) of cancer cells, although it is cytosolic in healthy mouse liver. Membrane-bound and cytosolic Bak both appear to be firmly bound to endogenous B-cell lymphoma-extra large (Bcl-XL) and are believed to undergo similar conformational changes and oligomerization in response to stress as Bax. Further, cytochrome c release is achieved by BID when it interacts via its BH3 domain, with the multidomain Bcl-2 family members Bax and/or Bak, causing their oligomerization and pore formation in the outer mitochondrial membrane. Due to this damage and pore formation, there is a potential release of cytochrome-c to the cytosol from the interior of mitochondria. The released cytochrome-c binds to apoptotic protease activating factor 1 (Apaf1), which is an adaptor protein, leading to the formation of Apoptosome, a protein complex that induces auto activation of initiator caspase-9. The active form of caspase-9 also cleaves and further activates caspase-3 to intensify the apoptotic signal response [79]. Apoptosis can be inhibited mainly due to the activities of Bcl-2 and Bcl-xL, which isolate Bax and prevent it from inflicting mitochondrial damage. Bcl-2 and Bcl-xL are common targets of the proapoptotic protein Bcl-2 antagonist of cell death (BAD), which specifically inhibits the activity of both antiapoptotic factors by forming heterodimeric complexes with any of the two proteins leading to displacement of Bax [80].

During an influenza infection, PB1-F2 plays a very important role by interacting with ANT3 and VDAC1 proteins at the inner and outer membranes of mitochondria (as shown in figure 8) [83]. VDAC1(voltage-dependent anion channel 1) acts as a gatekeeper allowing the passage of metabolites, nucleotides, and ions; it plays a critical role in regulation of apoptosis due to its interaction with apoptotic and anti-apoptotic proteins, namely members of the Bcl-2 family of proteins and hexokinase [81]. Adenine nucleotide translocase (ANT) plays a vital role in the exchange of ATP for ADP through the inner mitochondrial membrane, thus supplying the cytoplasm with newly synthesized ATP in oxidative phosphorylation [82]. As a result of this interaction, there is an enhancement in apoptosis.

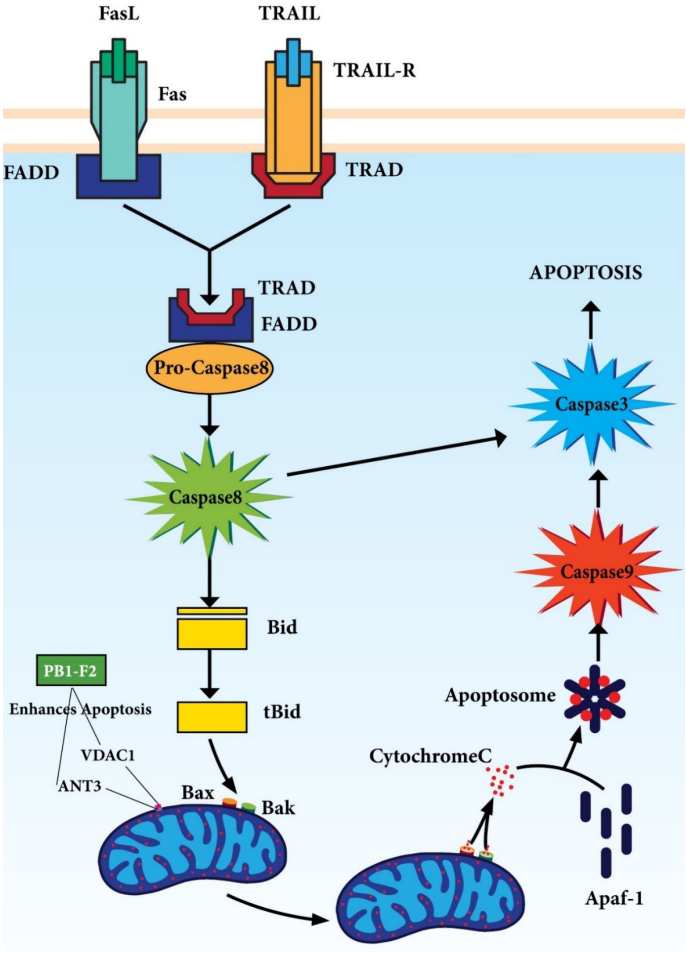

Figure 8: APOPTOSIS. Viral component PB1-F2 interacts with VDAC1 and ANT3 which are present in the inner and outer membranes of the mitochondria leading to enhanced apoptotic response.

Cell cycle

Cell cycle, or cell-division cycle, is a highly controlled process during which a cell undergoes duplication and division leading to the production of two daughter cells [84]. Based on studies of RNA viruses and cell cycle interaction, some positive-strand RNA viruses, namely, the coronavirus family, have been investigated extensively. Not much is known about other RNA viruses, specifically negative-strand RNA viruses. As a very significant negative-strand RNA virus, influenza virus which has caused global concern because of its antigenic variability and pandemic potential intensive research is currently being conducted on influenza. Previous studies have demonstrated that influenza virus is a cytolytic virus that encourages apoptosis in many cell types. Some viral proteins and many cell signaling molecules are found to be involved in induction of cell death during influenza virus infection. A common phenomenon of p53 activation in influenza virus-induced cell death has been observed in a variety of cell types. p53, a transcription factor which is important for various vital functions, not only is crucial in the apoptosis process but also plays a critical role in the regulation of cell cycle. The binding of activated p53 to DNA results in expression of p21, and subsequent binding of p21 to the cyclin E-Cdk2 complex inhibits the complex's activity and blocks the G_1/S transition leading to cell cycle arrest [85].

NS1 protein of influenza A virus is a non-essential viral protein with numerous additional functions during viral infection. It is seen that NS1 proteins promotes cell cycle arrest to further allow viral replication. Changes in the cell cycle signifies a series of complex and tightly controlled processes that mediate how cell division takes place. The first gap phase is regulated by the G_1/S cell cycle checkpoint. In this switch, two-cell-cycle kinesis, comprising CDK4/6-cyclin D and CDK2-cyclin E, along with the transcription complex, involving Rb and E2F, are keys to control this checkpoint. The Ras homolog gene family member A (RhoA) is a GTPase that mediates several cellular functions, including gene transcription, actin polymerization, cell cycle progression and cell transformation. RhoA exists in two forms, and the phosphorylation of Ser188 is central for its function in cell cycle shift. In the cell cycle, RhoA activation can affect G_1/S progression via three signaling pathways respectively. One deals with the repression of the RhoA-ROCK pathway leading to the build-up of INK4 family proteins and the competitive binding of CDK4 and CDK6. The second includes the down regulation of mDIA to increase the expression of $p21^{Waf1/Cip1}$ and $p27^{Kip1}$. And the third pathway showcases the effect of RhoA on extracellular signal-regulated kinase (ERK) activity to decrease the level of cyclin D1. Together, these three pathways reduce the levels of Rb phosphorylation, thereby inducing G_1/S cell cycle arrest [86].

Cyclin D3 is another important factor required for the cell cycle progression. It assists the G0/G1 cell cycle progression for which cyclin D3 has to be imported into the nucleus (as shown in figure 9). However, during an influenza A infection, there is an interaction between the matrix M2 protein of the virus and the cyclin D3. Due to this interaction, cyclin D3 gets trapped by the M2 protein thus hindering its transport into the nucleus where it is required for the progression of the cell cycle. As a result, cyclin D3 is forced to prevail in the cytoplasm where it undergoes proteasomal degradation. Thus, due to the unavailability of cyclin D3 G0/G1 cell cycle arrest occurs thus allowing the influenza virus to completely hijack the host cell and continue its viral infection [84].

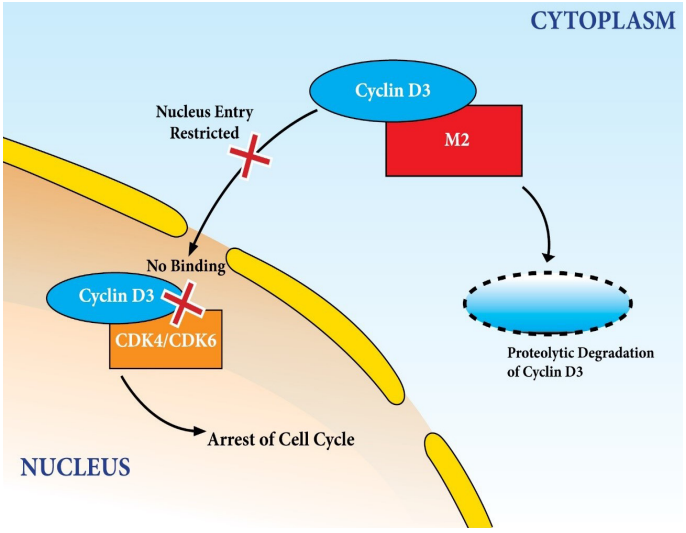

Figure 9: CELL CYCLE. Matrix protein M2 binds to cyclin D3 and does not allow its entry into the nucleus and due to prolonged exposure of cytoplasmic proteases cyclin D3 gets degraded. Since no cyclin D3 enters the nucleus there is no binding with CKD4/CDK6 and thus leading to cell cycle arrest.

mRNA surveillance pathway

NS1 protein of influenza A virus is a significant virulence factor that restrains host antiviral gene expression at various levels in the nucleus [87]. In addition to the major deadenylation-dependent mRNA decay pathway, cytoplasmic mRNAs also undergo specialized endonuclease dissociation, triggered by endonuclease cleavage of mRNA through cellular or viral endonuclease and then cellular exosomes and XNR1 for exonuclease digestion [88]. Xrn1 commutes between the cytoplasm and the nucleus along with other components of the mRNA decay pathway that rely on deadenylation, where they bind to the transcription start sites and directly mediate the initiation and elongation of genes. The transport of Xrn1 and other decay factors to the nucleus depends on the exoribonucleolytic activity of Xrn1. By linking mRNA synthesis to decay, Xrn1 maintains mRNA homeostasis, since the defects in the breakdown of 5'-3' mRNA decay are buffered by a decrease in mRNA synthesis [89]. Two mRNA surveillance pathways monitoring cellular mRNA quality are nonsense-mediated decay (NMD) and no-go decay, which uses the endonucleotide decay mechanisms to ensure perfect gene expression. NMD targets mRNAs with premature stop codons and mRNAs with abnormally long 3'UTRs, whereas no-go decay removes mRNA with secondary structures of delayed ribosomes. On the other hand, another mRNA surveillance pathway called nonstop decay targets mRNA without a stop codon for exonuclease degradation of SKI(Ski2-Ski3-Ski8) complexes and exosomes [88].

This impact incorporates mRNA processing, which is intervened by the association of NS1 with the cleavage and polyadenylation specificity factor (CPSF30/CPSF4), the nuclear poly(A) binding protein (PABPN1) (otherwise called PABII) and a putative splicing factor (NS1-BP) [90,91]. The binding of NS1 to CPSF and PABII inhibits polyadenylation of the host mRNAs, imparting to the nuclear retention of these messages. Yet another interaction takes place with the chromatin remodelers such as PAF1 (The polymerase-associated factor 1), and constituents of the mRNA export machinery, including NXF1 (nuclear export factor 1), mostly in a consecutive or co-ordinated way as the host mRNAs are being prepared and exported subsequently [91]. The mRNA export receptors NXF1-p15 (TAP-NXT) are key

constituents of the mRNA export machinery that is liable for the nuclear exit of about 70% of the cellular mRNAs. This heterodimer cooperates with both messenger ribonucleoprotein particles and nuclear pore complex proteins [nucleoporins (Nups)] to direct mRNAs through the nuclear pore complex (NPC). Another factor named E1B-AP5, recognized as a cellular protein that connects with the adenovirus protein E1B-55 and involved in mRNA export, is a (Heterogeneous nuclear ribonucleoprotein) hnRNP-like protein that presumably intervenes the interaction of NXF1 with mRNAs. Furthermore, other RNA-restricting proteins like the REF family and SR (Serine/arginine-rich) splicing factors are known as adaptors for the association among NXF1 and mRNAs. Besides, the mRNA export factor Rae1/mrnp41/Gle2 which transports between the core and the cytoplasm, shapes a complex with RNPs, NXF1, and the nucleoporin Nup98 (as shown in figure 10). Rae1 may direct TAP towards Nup98 to intervene transport through the NPC [90].

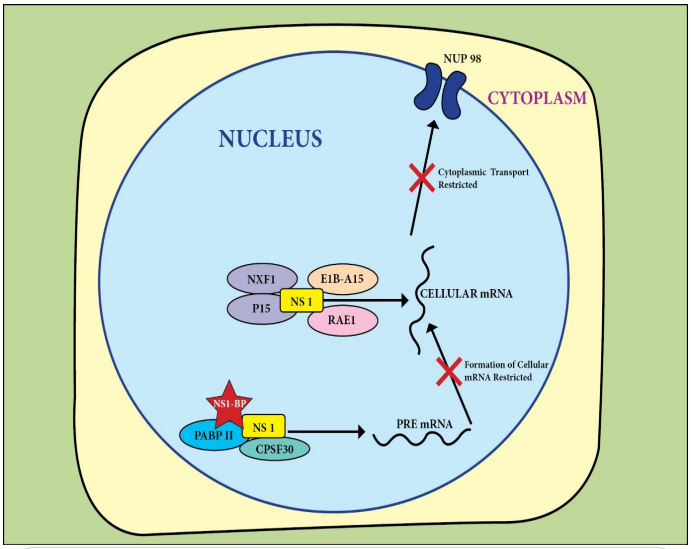

Figure 10: mRNA Surveillance pathway A] The interaction of NS1 protein with the complex comprising the cleavage and polyadenylation specificity factor (CPSF30), the nuclear poly A binding protein (PABPII) and a putative splicing factor (NS1BP) inhibit the polyadenylation of the host RNAs. B] NS1 interacts with the mRNA export machinery inhibiting the transport of cellular mRNA into the cytoplasm via NUP98 Nucleoporin.

RNA transport

The nuclear transport of cellular mRNA is intervened by various proteins binding to mRNA and the pre-mRNA precursors. Nonetheless, dissimilar to cellular intron-containing mRNAs, most influenza virus mRNAs are intron-less. Subsequently, the transport pathways for the viral intron-less mRNAs may be different from those of the cellular mRNAs. In addition, as there are three different sorts of influenza virus mRNA, more than one system of nuclear export may work in virus-infected cells. The first sort of influenza virus mRNA incorporates intron-less mRNAs like PA, PB1, PB2, HA, NA, and NP mRNA. The second sort of viral mRNA comprises the M1 and NS1 mRNAs, which contain introns, however, do not undergo splicing. The M2 and NS2 mRNAs, which are created by splicing, involve the third kind of viral mRNA [92]. vRNA interacts with a heterotrimeric polymerase complex comprising of PB1, PB2 and PA proteins along with NP to frame a vRNP [93].

Nuclear transport of vRNPs is known to happen in CRM1-dependent accordance. CRM1 (otherwise called Exportin1/XPO1) is a cellular transport protein that, along with Ran GTPase, is liable for the transport of cellular cargo across the nuclear pore complex (NPC). It perceives cargo proteins that have leucine-rich nuclear export signals (NESs). vRNP accesses the nuclear export pathway through chromatin, the site of RanGTP recovery from RanGDP, a cycle catalyzed by the guanine trade factor Rcc1. The widely known model for nuclear export of vRNPs includes partaking of the viral matrix protein 1 (M1) and the nuclear export protein (NEP) (otherwise called nonstructural protein 2, or NS2) in establishing the relationship of CRM1 with vRNPs in an alleged daisy chain arrangement. In this model, CRM1 identifies two NESs close to the N terminal of NEP, while a C-terminal clip of NEP partners with an N-terminal nuclear localization signal of M1. Thus, M1 ties to the vRNP through C-terminal cooperation with the NP constituent of vRNPs. This model quite recently has been refined to incorporate a role for the viral RNA-dependent RNA polymerase. Specifically, NEP has been seen collaborating with the PB1 and PB2 polymerase segments of the vRNP, and this communication among NEP and the polymerase is proposed to assist the cooperation of M1 with NP. The interaction with the promoter RNA disrupts the conformation of the heterotrimeric viral RNA polymerase; accordingly, the RNA polymerase could be available in different frameworks with respect to vRNPs and complementary RNPs (cRNPs), the replicative intermediates of the influenza virus RNA genome [93]. The nuclear export of RNPs is a directed interaction, with various separate mechanisms promoting the export at late time points in infection. The advancement of the infection triggers the apoptotic pathway, encouraging RNP transport through the activation of caspase 3, as the activated caspase 3 promotes the dissemination limit of nuclear pores.

Aggregation of the viral surface protein HA at the cellular surface, late in infection initiates the mitogen-activated protein kinase (MAPK) signaling cascade, which enhances the RNP transport. Nuclear export transports RNPs to the perinuclear cytoplasm, where they assemble at a region containing the microtubule coordinating focus (MTOC) and the aggregation of RNPs here might be clarified by Y-box Binding protein 1 (YB-1), which binds to RNPs in the core and interact with microtubules after nuclear export. In the proximity of MTOC, RNPs cooperate with recycling endosomes (REs) through Rab11 [94]. After vRNPs leave the nucleus, they are assumed to hitch a ride (travel onto) on Rab11-containing endosomes. Rab11 is a group of small host cell GTPases engaged with apical trafficking of recycling endosomes (REs) and consists of three members (Rab11a, Rab11b, and Rab25) in mammalian cells [93]. Intracellular transport of vRNP from the nucleus to the plasma membrane is thought to be mediated by Rab11A via direct protein-protein interaction with the PB2 subunit of the polymerase. Rab11A specifically marks recycling endosomes (RE), which sort and transport cargo slated for release from the apical cell membrane, recruits various molecular motors to RE through interactions with its corresponding interacting family (Rab11-FIPs) [95], and co-localizes with vRNA sections and NP. Considering the type of cargo and the distance to be travelled, tubular REs can utilize both actin and microtubules for transport by means of various motor proteins. Rab11-family-interacting proteins (FIPs) are thought to intercede interaction with the host cytoskeleton through direct interaction with motor proteins. Rab11a-containing REs exhibit movement along the actin cytoskeleton by means of FIP2 and myosin Vb motor protein, and along microtubules through FIP3 and dynein (or kinesin superfamily, or KIF, protein) motors. The variety in motor protein utilization during Rab11a RE movement may clarify how vRNPs could be transported on microtubules as well as actin, (as shown in figure 11) [93].

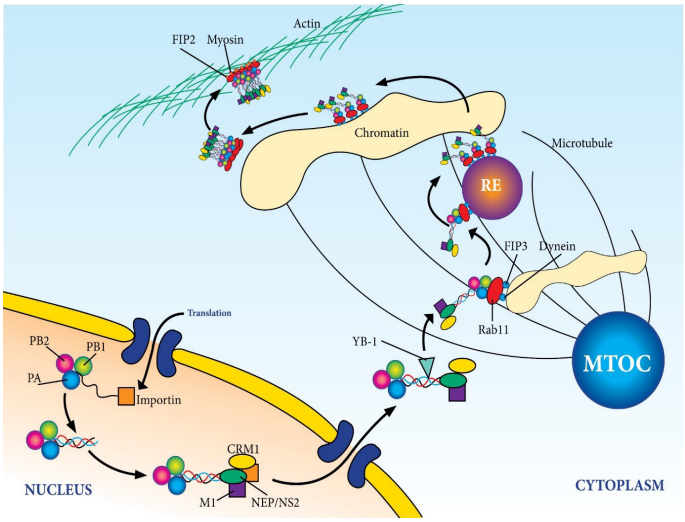

Figure 11: RNA transport mechanism. The viral proteins (PB1, PB2 and PA) translated in the cytoplasm are imported into the nucleus via importin, which then assembles along the nucleoprotein and forms the viral ribonucleoprotein (VRNPs). CRM1 along with the RanGTPase, M1 and NS2 protein transport the VRNPs into the cytoplasm forming a Daisy chain arrangement. The VRNPs are transported to the plasma membrane through Rab11-RE interaction via actin and microtubular cytoskeleton.

Mitogen-Activated Protein Kinase (MAPK) signaling pathway:

The initiation of the immune response towards influenza infection, like other viral infections, is supported by the initiation of various molecular pathways including IRF3, NFκB, and the MAPK pathways. Dysregulation of these pathways may bring about overly produced immune responses like immune cell overactivation and inflammatory cytokine overproduction known as the "cytokine storm". These are usually connected with deterioration in infection and are to be considered as good targets for immunomodulatory treatments.

The MAPKs, a group of proline-coordinated, protein-serine/threonine kinases, are evolutionary moderated cell regulators that pass on extracellular signs as phosphorylation falls to get focused on intracellular reactions [96]. Mammalian MAPKs comprise four unique subgroups being: extracellular-signal-regulated kinase 1 and 2 (ERK1/2), c-Jun N-terminal kinases/stress-activated protein kinases (JNK/SAPK), p38 MAPK, and ERK5/Big MAP kinase 1(BMK1) [97]. Comprehensively, growth factors and mitogens activate ERK1/2 and ERK5, while cellular stress signals and inflammatory cytokines trigger both JNK and p38. Upon extracellular incitement, MAPKs are triggered downstream of successively initiated protein kinases: MAPK Kinase Kinase (MKKK) and MAPK kinase (MKK). These kinases direct the activities of various cytosolic and nuclear proteins, controlling different cell exercises including actuation, propagation, distinction, effector functions, and apoptosis [96]. MAPK signaling advances cell endurance by double phosphorylation on threonine and tyrosine residues. The upstream MAPKK regulates the enzyme activities. The 2 MAPKK (MKK3/6, MKK4/7) are liable for the triggering of p38 and JNK, individually. These proteins are associated with apoptosis and cytokine expression and can be enacted by ecological stress conditions. The upstream Raf controls the phosphorylation of MAPK/ERK kinase (MEK) ½, which directs the triggering of ERK ½, playing a regulatory role in cell expansion and distinction. And the compound ERK5 is initiated by MEK5. Influenza infection activates all individuals from the MAPK family, which are believed to advance vRNP traffic and virus production.

JNK initiation is prompted by the aggregation of RNA created by viral polymerase. Early-stage of infection initiates the activator protein-1 (AP-1), a transcription factor that incorporates c-Jun and ATF-2, whose transcriptional activity is upgraded by JNKs, a part of the MAPK signaling pathway. AP-1 contributes to the expression of interferon-β (IFN-β) and antiviral cytokines as well. Moreover, inhibition of JNK by predominant negative mutants of MKK7/JNK/c-Jun brings about hindered transcription from IFN-β promoter during influenza infection, resulting in increased viral production. Subsequently, this pathway is significant as a middle person of an inflammatory response to the influenza infection by co-directing IFN-β expression. P38 MAPK initiation regulates the expression of RANTES (Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted, also called CCL5- Chemokine ligand 5) creation and chemokines by influenza infection. In profoundly pathogenic H5N1-infected cells, p38 initiates tumour necrotic factor (TNF) cytokine. It is also known that IL-1β animates activation of p38 MAPK with prostaglandin E2 production. The p38 inhibitor diminishes the release of prostaglandin E2 and furthermore lessens the virus titre, which proposes that p38 MAPK actuation is fundamental for inflammatory response and adds to the viral replication procedure. Influenza infection instigates TNF-α in a p38-dependent way.

Influenza infection upregulates Raf/MEK/ERK pathway and MAPK Signaling which is significant for the productive export of nuclear RNPs. The inhibition of MEK obstructs this pathway which appeared to hinder the growth of the virus and decrease the transport of the vRNP. Strangely, it doesn't influence viral RNA or protein synthesis. This implies that the nuclear RNP transport shows up in the inducible stage and relates well with the theory that ERK activation happens in the late period of infection. It is also suggested that Raf/MEK/ERK signaling is initiated by legitimate aggregation of HA/lipid-raft complex inside the cellular membrane. Impaired transport of HA from the cytoplasm to the cell surface, could be a potential justification for the low initiation of ERK. The viral polymerase complex is directly accountable for the aggregation of HA with connection to the MAPK signaling, supporting that more ERK activation follows more proficient nuclear RNP transport and expanded production of progeny virions, (as shown in figure 12) [97].

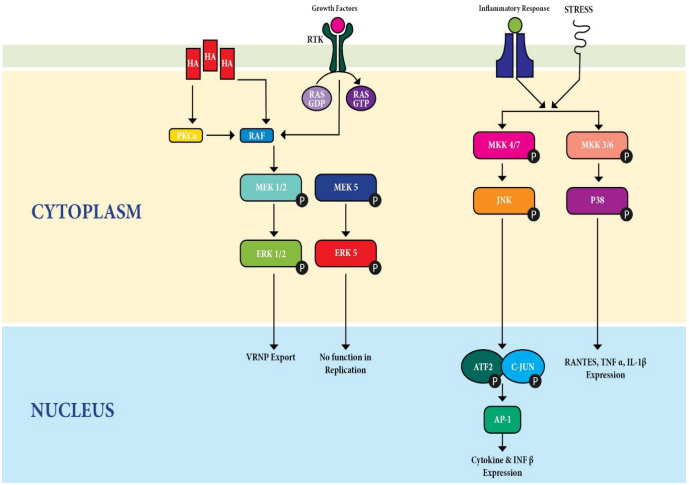

Figure 12: MAPK signaling pathway. The Raf/MEK/ERK signaling is initiated by aggregation of HA/lipid-raft complex inside the cellular membrane. This leads to the activation of PKC α, an upstream molecule of Raf, signaling the autophosphorylation of the MEK1/2 followed by ERK 1/2 resulting in the enhancement of vRNP transport through the nucleus. Simultaneously, the autophosphorylation of Raf gene is also triggered via RTK-RAN complex (GDP phosphorylation).

Budding, Packaging and Release

Enveloped viruses show the penultimate and final steps of their replication which are assembly and budding, before the release of infectious virion progenies. Lytic viruses, as influenza, do not form a long-term, stable, virus-host relationship within the infected host. Thus, are dependent highly on host-to-host transmission, which in turn depends on the release of the virus from the progeny of the infected host. And because full-length influenza viruses are not present in infected cells and can only be produced by budding through the plasma membrane, assembly, as well as the budding processes, pose a critical role in the production of infectious viruses, which in turn affects both the severity of the disease and the virus transmission between humans and animals playing the role of carriers.

During assembly, the viral components produced in various cell compartments are brought to the budding site on the host cell's plasma membrane. Surface glycoproteins of viruses such as influenza virus, parainfluenza virus, or respiratory syncytial viruses (RSV), which sprout from the apical plasma membrane, have apical sorting signals and accumulate in cells predominantly on the apical plasma membrane [98]. IAV envelopes are fortified with cholesterol and sphingolipids, suggesting that they come from different regions of the apical plasma membrane, often referred to as "rafts". It is believed that HA is localized in these different regions based on fatty acid modifications of the C-terminal cysteine found in the Golgi apparatus, whereas the enrichment of NA was ascribed to a property in the C-terminus of TMD (transmembrane domains). In contrast, M2 has been shown to accumulate onto the edges of these budding domains, and it has been suggested that the cytosolic protein M1 is localized in the budding region associated with the short cytoplasmic tails of HA and NA. It is plausible that NA and HA produce membrane domains with unique lipid profile that have a high affinity for M1 at the periphery of Rab11, where they localize at the bud site by binding to M1, in addition to orchestrating the correct viral components at the apical bud site; IAVs must also reshape the membrane to induce bud formation and ultimately, cleavage of the viral envelope from the plasma membrane. To encourage budding, the virus must first induce significant curvature in the membrane and then contract the two opposing sides of the plasma membrane causing the viral envelope to facilitate membrane rupture. Curvature can be induced by (i) protein or "molecular" stacking in a leaflet of a bilayer, (ii) association of curved or "bent" proteins with the bilayer, (iii) biased conical accumulation of lipids in a leaflet of the bilayer or (iv) the cytoskeleton. Expression of HA and NA is sufficient to induce budding and this efficiency and uniformity of shape are benefitted by the presence of M1. This suggests that the frequency of HA and NA on one side of the membrane may contribute to the curvature. The asymmetrical shape of NA also plays a role here, since clumping of the virus membrane is often observed. In contrast, M1 appears analogous to a membrane diffractive protein which when recruited to the cytosolic side of the membrane budding site, is oligomerized upon reaching the membrane, these oligomers have been molded to form curved structures. In these properties, it is plausible that M1 significantly affects the curvature of the membrane at the budding site, possibly explaining its role in distinguishing whether IAV forms spheres or filaments. The M2 ion channel is on the border of the budding site contributing to IAV cleavage by acting as a membrane flexing protein, located on an amphiphilic α-helix that can incorporate the amino acid side chains on its hydrophobic side into a leaflet of the bilayer, which when positioned in the cytosol, results in negative curvature of the membrane through intercalation. This has been proposed to facilitate the formation of the viral stem neck and the cleavage, presumably by reducing the distance between the two opposing membranes of the virus envelope [4].

Budding is the process that leads to the formation, growth, and release of virus progenies in the form of buds. Although the structure and release of influenza virus progenies have evolved for the survival of virus species, both infectious virus particles and non-infectious or defective virus particles are produced. The presence of a segmented RNA genome further complicates the whole process. Morphogenesis and budding require four steps: assembly of the viral components, yolk initiation, yolk growth, and clamping of the plasma membrane. These steps in the budding process are sequential and have no specific start and stop signals. During the assembly process, the viral components, either individually or in the form of complexes, are brought to the budding site to form a higher order complex, facilitating the start and /or completion of the bud. For most enveloped or non-enveloped viruses, the assembly involves the formation of the entire capsid, either helical or icosahedral, including the incorporation of the genome into the capsid. However, the requirements for capsid assembly and budding are much more complex for the influenza virus for several reasons: First, budding can occur without vRNPs and /or with incomplete vRNPs. In addition, the viral genome consists of several vRNA / vRNP segments. Therefore, the budding of an infectious viral particle requires each segment of vRNP to be incorporated into the bud. Second, all the components of the virus, i.e., an envelope containing the transmembrane protein (HA, NA and M2), as well as M1 and vRNP, individually or as a complex, ought to be brought to the bud site in order to start the bud formation: its growth and finally the release of the infectious virus buds. Influenza virus particles contain three subviral components, namely (a) a viral envelope consisting of a lipid bilayer and viral transmembrane proteins (HA, NA and M2). Lipids are selectively derived from the host. (b) M1 protein under the lipid bilayer, which forms the bridge between the envelope and the viral core. (c) Virus core (viral nucleocapsid), consisting of vRNP (minus strand vRNA and NP) and small amounts of NEP and 3P protein complex (polymerase). The NP monomer forms a hairpin structure and the vRNA is exposed on the outer surface of the NP. Therefore, assembly involves the formation of these subviral complexes and their transport to the budding site, the apical domain of the plasma membrane in the polarized epithelium.

The following viral component complexes have been identified in infected cells or virions and are likely to play an important role in virus assembly and some also in the budding process: HA - HA (trimer), HA - M1, NA - NA (tetramer), NA - M1, M1 - M1 (multimer), M1 - M2, M1 - NP, M1 - RNA, M1 - RNP, M1 - RNP - NEP, NP - RNA, PB1 - PB2 - PA (3P), 3P- RNP, 3P-RNP-M1, PB1 / PB2-NP. Two models are supposedly used for incorporating eight vRNA / vRNP segments into virions: “Random Packaging” and “Specific Packaging”. The "Random Packaging" model includes the presence of common structural elements in all vRNPs, which leads to the fact that they are randomly incorporated into virions and therefore the incorporation of vRNP is concentration dependent. On the other hand, the “specific packaging” model is based on specific structural features that are present in every vRNA / vRNP segment, which means that they can be incorporated into virions in a targeted manner.

Bud formation and bud discharge require the gathering of viral components, which could arise both during transportation or in the course of the process of budding on site. The budding system itself calls for 3 predominant steps: bud initiation, bud propagation, and bud completion freeing the virus from the host's cellular membrane. Each of the steps includes the interplay of various host and viral components: Bud initiation calls for outward bending of the plasma membrane and includes the transition of a planar membrane shape to a curved shape on the budding site. Each lipid rafts and raft-related protein on the budding site pose a critical function in inflicting membrane curvature and bud initiation. M1 interacting with the internal leaflet of lipid bilayers is possibly to play an essential function in bud initiation. Clustering of M1 because of M1/M1 interplay under the lipid bilayers can purpose outward membrane bending and bud initiation.

Bud growth/ propagation

This leads to bud maturation and is the intermediate level among bud initiation and bud discharge and also determines the dimensions and the morphology of discharged virus progenies. Influenza viruses are distinctly pleomorphic, and the dimensions of the discharged progenies can range from spheroidal, elongated, or even filamentous and the content material of the nucleocapsids isn't always the predominant aspect for bud growth. Influenza virus bud increase seems alternatively dependent upon forces, a pulling and a pushing pressure. The pulling pressure is normally supplied through the transmembrane proteins alongside M1 which can be pulling nucleocapsids into the bud. On the alternative hand, the cortical actin microfilaments which bind to viral RNPs offer the pushing pressure for incorporating the nucleocapsids and M1 into the bud.

Bud closure

This is the very last step for the scission of the bud and launch of the virus particle into the outer environment. Bud closure results in the fusion of opposing terminals of the viral membranes. This might require bringing and maintaining the opposing membrane ends after every other end in near proximity so that it can discover its counterpart inflicting fusion of corresponding lipid bilayers. Virus buds might then turn out to be separated from the membrane of the parent infected cell. This shows that lipid bilayers are to be held in very near proximity for the fusion to arise. Host and viral elements may have effective and poor influences on bud discharge [98].

The release is highly dependent on the sialidase activity of NA. NA is a homotetramer and each subunit consists of a short N-terminal cytoplasmic tail (six amino acids) followed by a TMD, a variable-length stem and a globular enzyme head domain. The globular head domain consists of a 6-bladed propeller structure, where each blade is comprised of four antiparallel β-sheets stabilized by disulfide bonds. The catalytic Tyr residue is found in a highly conserved active site which creates a profound pocket in the centre of each monomer. All the residues necessary for catalysis are present in every monomer, which makes it difficult to explain the evolution of NA into a tetramer. Calcium and calcium ions have been shown to contribute to NA activity. NA enables virus release by catalysing the hydrolysis of the glycosidic bond that binds SA to the underlying sugar molecules. By removing local SA residues, NA prevents HA from binding to the cell surface, thereby facilitating virus release during budding. It has been shown to promote IAV cleavage by removing SA residues from N-linked glycans that are on HA and NA molecules in the viral envelope [4]. Splitting off the influenza virus buds from infected cells is the final step in completing the virus' life cycle. This step seems to limit the speed, as many mature virus particles stick to the cell membrane and only a relatively small part of the virus buds, (~10%) are released [98].

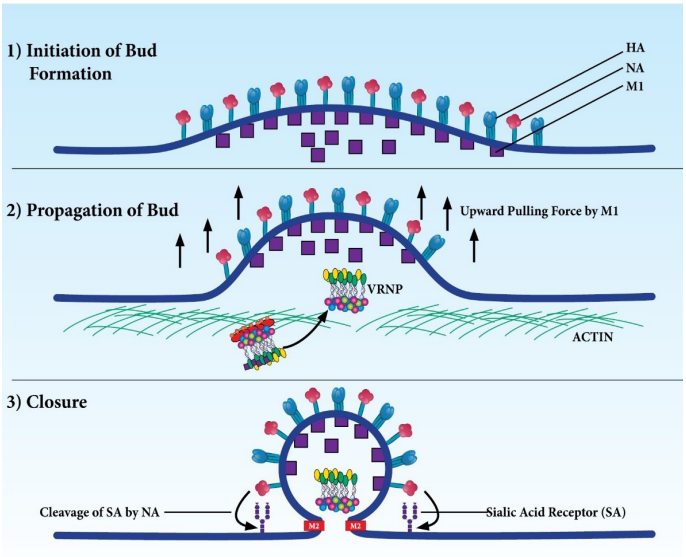

Figure 13: Budding mechanism of Influenza A virus.

Conclusion

It is profoundly established that the host immune response to influenza A virus infection includes numerous complex processes that harmonize together to play effective roles towards protecting the host. Novel approaches are essential for the establishment of effective treatment, therapeutics and vaccine development. While our understanding of influenza A virus-host interaction has increased greatly, extensive studies are required for a better understanding of host immune system mechanism upon detection of the virus. During the study, we came across the fact that the Non-structural protein 1 (NS1) of the influenza A virus plays a very important role in regulating the majority of the immunological signaling pathways during the infection. This implies that there is a need for NS1 targeted treatment against influenza A virus infection. Further in vitro/in vivo studies should be addressed for the same. The main goal would be to use these methods in the discovery of novel therapeutics and ultimately to the development of a universal influenza vaccine.

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