Influenza A virus PB1-F2 protein: An ambivalent innate immune modulator and virulence factor

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Abbreviations: AIV, Avian influenza A virus; ANT3, adenine nucleotide translocase 3; AP1, activator protein 1; ARDS, acute respiratory distress syndrome; CARD, caspase activation and recruitment domain; Csf3, colony-stimulating factor 3; Cxcl, chemokine (C-X-C motif) ligand; DDX3, DEAD-box RNA helicase 3; ΔΨm, mitochondrial membrane potential; IAV, influenza A virus; IFN, interferon; IKK, IκB kinase; IRF3, interferon regulatory factor 3; MAM, mitochondria-associated endoplasmic reticulum membrane; MAVS, mitochondrial antiviral-signaling protein; MOI, multiplicity of infection; mPTP, mitochondrial permeability transition pore; Mtn1, mitofusin-1; mtROS, mitochondrial reactive oxygen species; NDP52, nuclear dot protein 52; NLRP3, NACHT, LRR and PYD domains-containing protein 3; NLRX1, NLR family member X1; OMA-1, overlapping with the M-AAA protease 1; OPA-1, optic atrophy protein 1; PACT, protein activator of PKR; PB1, polymerase basic 1; PB1-F2, polymerase basic 1 frame 2; RIG-I, retinoic acid-inducible gene 1; STING, stimulator of interferon genes; TBK1, TANK-binding kinase 1; Tom40, translocase of outer mitochondrial membrane 40; TRAF6, tumor necrosis factor receptor associated factor 6; Trem1, triggering receptor expressed on myeloid cells 1; UPS, ubiquitin proteasome system.

Abstract

Influenza A virus (IAV) causes not only seasonal respiratory illness, but also outbreaks of more severe disease and pandemics when novel strains emerge as a result of reassortment or interspecies transmission. PB1-F2 is an IAV protein expressed from the second open reading frame of PB1 gene. Small as it is, PB1-F2 is a critical virulence factor. Multiple key amino acid residues and motifs of PB1-F2 have been shown to influence the virulence of IAV in a strain- and host-specific manner, plausibly through the induction of apoptotic cell death, modulation of type I interferon (IFN) response, activation of inflammasome, and facilitation of secondary bacterial infection. However, the exact role of PB1-F2 in IAV pathogenesis remains unexplained. Through reanalysis of the current literature, we redefine PB1-F2 as an ambivalent innate immune modulator that determines IAV infection outcome through induction of immune cell death, differential modulation of early- and late-type I IFN response, and promotion of pathogenic inflammation. PB1-F2 functions both intracellularly and extracellularly. Further investigations of the mechanistic details of PB1-F2 action will shed new light on immunopathogenesis of IAV infection.

KEYWORDS

Influenza A virus, innate antiviral response, type I IFNs, inflammation, inflammasome, avian influenza virus

1. Introduction

Influenza virus is an enveloped virus belonging to the family of *Orthomyxoviridae* with a single stranded, negative sensed and segmented RNA genome.[1] Symptoms of influenza range from mild respiratory illnesses such as sore throat, runny nose, muscle pain and mild fever to severe conditions including high fever, acute respiratory distress syndrome (ARDS), multi-organ failure, secondary bacterial infection or even death.[2-5]

Among the four genus of influenza virus A to D, influenza A virus (IAV) is most virulent to humans. Since early nineteenth century, there have been five IAV pandemics, claiming millions of lives globally.[6] Unlike other genera, IAV adapts to multiple nonhuman reservoir species such as birds, pigs, horses, cows, bats as well as domestic pets including cats and dogs.[7, 8] High pathogenicity strains can emerge when IAV crosses species barrier to infect humans. Typical examples are H5N1 and H7N9 avian influenza A viruses (AIVs) emerged in 1997 and 2013, with a high case fatality of 55% and 40%, respectively, in humans.[9, 10] In addition, 24 cases of human infection with H5N6 AIV have been reported from China since 2014, including 7 deaths.[11]

Contrary to the general belief, cross-species infection of humans with AIVs could also be mild. For example, H7N7 and H9N2 AIVs occasionally infect humans but cause mild diseases in most cases, resembling human seasonal IAVs.[12-15] This indicates that virulence of IAV depends not only on the host including pre-existing immune memory, but also on the virus including virulence factors, which are accounted for increased pathogenicity due to facilitation of viral entry and replication, evasion of host antiviral immunity, dysregulation of inflammatory response and direct cytotoxicity.[16]

Among all IAV virulence factors, PB1-F2 is unique and multifaceted. In 2001, it was discovered as an “immune cell killer”, which induces apoptotic death of immune cells.[17, 18] Infection

studies in different animal models reveal species- and strain-specific pathogenicity of PB1-F2-defective IAVs.[19-24] Opposite to the observations in mammals that loss of PB1-F2 often renders IAV less pathogenic,[19, 25] expression of PB1-F2 results in the attenuation of the virus but extension of virus shedding in chickens.[21, 22] Whether and to what extent this might be attributed to the interaction and competition between PB1-F2 and HAX-1, an IAV restriction factor that inhibits PA subunit of viral polymerase remain to be elucidated.[26, 27] It is known that chicken have NLRP3 but not AIM2.[28, 29] However, it remains unclear how defects in the activation of inflammasome pathways in chicken might affect PB1-F2 function. By and large, growing evidence accumulated in the past two decades supports the notion that PB1-F2 affects the outcome of IAV infection by modulating host innate immunity both positively and negatively. The delicate balance of antiviral response and inflammation in the presence of PB1-F2 has important implications in viral pathogenesis and disease intervention. The evolutionary conservation of PB1-F2 and the evidence that PB1-F2 sequence is under strong selection pressure[30, 31] lend support to the importance of PB1-F2. In this review, we summarize the current knowledge of PB1-F2 protein and the mechanism by which PB1-F2 perturbs innate immunity. We also provide an overall model to explain the action of PB1-F2 as an ambivalent innate immune modulator and virulence factor.

PB1-F2 protein is the eleventh IAV protein discovered through the characterization of PB1-F2-targeting $CD8^+$ T cells.[17, 32] Indeed, PB1-F2 is immunogenic[17, 33, 34] and anti-PB1-F2 antibodies were found to contribute to protection in mice.[35] Yet, PB1-F2 is a non-structural protein not found in the IAV virion.[36] PB1-F2 is expressed from the +1 open reading frame with respect to PB1 gene on segment 2 of the IAV genome. As the result of a frame shift, PB1-F2 is produced as a completely different protein compared to PB1, which is a structural protein subunit of viral polymerase. Translation of PB1-F2 is likely initiated through leaky ribosome scanning under the control of elements downstream of the initiation codon.[37, 38] In stark contrast to PB1, PB1-F2 is a small viral

protein with 87 to 90 amino acid residues in full length and localized predominantly to mitochondria.[17, 39] PB1-F2 targets mitochondrial inner membrane facing intermembrane space.[40, 41] The mitochondrial targeting sequence of PB1-F2 is in the C terminal region between residues 65 and 87 and assembles into a positively charged α-helix structure.[40] Tom40 was recently found to be a necessary adaptor in mitochondrial localization of PB1-F2.[41]

2. PB1-F2 – an immune cell recruiter and killer

PB1-F2 protein is too small to carry functional enzymatic domain. It was originally found to promote apoptotic cell death by permeabilizing mitochondrial membrane.[17] Mechanistically, PB1-F2 interacts with subunits of mitochondrial permeability transition pore (mPTP) complex VDAC1 and ANT3 to activate permeability transition that enhances apoptosis during IAV infection.[18] Moreover, PB1-F2 protein can form channel-like pore by self-aggregation on mitochondrial membrane to directly mediate leakage of mitochondrial content such as cytochrome C to initiate intrinsic apoptosis.[42, 43] Indeed, structural analysis reveals that PB1-F2 forms amyloid β aggregate in membranous environment.[44, 45] PB1-F2 protein changes from monomer to higher-order oligomer or protein aggregate during lytic IAV life cycle, more rapidly in U937 monocytic cell line than A549 lung cell line.[46, 47] In line with this, PB1-F2-mediated apoptotic response is more pronounced in immune cells, such as monocytes and macrophages.[17, 32, 48, 49] Plausibly, rapid protein aggregation of PB1-F2 quickly forms pores over mitochondrial membranes to trigger exaggerated apoptotic responses in these cells. In contrast, slower aggregation of PB1-F2 in A549 cells merely activates mPTP, which primes cells for minimal apoptosis.[18, 50] Moreover, PB1-F2 can be phosphorylated by protein kinase C at T27 and S35. This phosphorylation is required for the proapoptotic function of PB1-F2 in

monocytes.[51] Although detailed underlying mechanism of PB1-F2-mediated apoptosis in immune cells remains largely elusive, PB1-F2 is characteristic of an "immune cells killer".[48]

Immune cells such as phagocytes play important roles in antiviral immunity against IAV and viral clearance.[52-54] NLRX1 was recently identified as an anti-apoptotic protein to PB1-F2 in macrophages.[49] NLRX1 was required for macrophage survival and antiviral activities such as type I IFN production in response to IAV infection.[49] Knocking out NLRX1 enhanced macrophage apoptosis, reduced type I IFN production and suppressed virus clearance. Mechanistically, NLRX1 targets and binds to PB1-F2.[49] Indeed, apoptotic cell death induced by PB1-F2-deficient IAV was unaffected by NLRX1 knockout, although the phenotype of cell death attributed to PB1-F2-deficient IAV was less robust than that ascribed to the wild-type IAV counterpart. This indicates the specificity of NLRX1 to PB1-F2 in suppressing apoptosis and supporting macrophage-mediated antiviral function.[49]

PB1-F2-mediated apoptosis is IAV strain-specific. Two early reports claimed that only PB1-F2 of PR8 H1N1 possessed proapoptotic properties.[43, 55] However, subsequent study showed that PB1-F2 of AIVs H5N1, H6N1 and H2N3 but not mammalian H1N1 promoted apoptosis in porcine macrophages.[56] Interestingly, PB1-F2 of 1918 H1N1 of the Spanish flu was shown to be[57] or not to be[43] proapoptotic in different experimental settings such as viral backbone and cells. Although it is still unclear which specific residues of PB1-F2 are required for the strain-specific proapoptotic effect, it is plausibly governed by properties such as PB1-F2 binding affinity to mPTP and NLRX1 as well as its pore-forming capability through self-aggregation. PB1-F2 is also known to be an inhibitor of natural killer (NK) cells.[58]

In addition to being an immune cell killer, PB1-F2 is also an "immune cell trap" that attracts immune cells to the site of infection as a result of proinflammatory response. It was found that PB1-

F2 expression increased both pulmonary leukocyte infiltration and cell death in IAV-infected mice.[59] Transcriptomic study found that PB1-F2 promoted expression of chemokines such as Csf3, Cxcl3, Trem1 and Cxcl2, which attract leukocytes such as neutrophils and monocytes to infected lung tissue.[58, 59] By using *in vivo* κB reporter assay, it was shown that PB1-F2 strikingly enhances NF-κB activity in infected lung.[59] Indeed, enforced expression of PB1-F2 directly activates NF-κB.[60] NDP52 protein, an autophagy adaptor that physically interacts with PB1-F2, and TRAF6 protein are also implicated in PB1-F2-mediated NF-κB activation.[61] Together with its proapoptotic property, the proinflammatory nature of PB1-F2 should also be influential on overall IAV virulence. Indeed, when PB1-F2 induces chemokine expression and consequent leukocyte infiltration, more leukocytes are susceptible to IAV infection and then killed by PB1-F2 through apoptosis.[59]

3. Extracellular PB1-F2 – NLRP3 inflammasome activation and necrosis

PB1-F2 can function extracellularly to elicit lethal inflammation. It was found that direct intranasal application of peptide corresponding to 27 amino acid residues of PB1-F2 C-terminus alone was sufficient to elicit severe immunopathogenic effect and secondary bacterial infection, leading to higher mortality in mice.[43, 62-64] Indeed, PB1-F2 peptide was found to activate NLRP3 inflammasome in macrophages.[65] Mechanistically, it was found that extracellular PB1-F2 peptide formed protein aggregate of over 100kDa in size and macrophage phagocytosis is required for internalisation of PB1-F2 peptide for NLRP3 inflammasome activation.[65] Caspase 1 is cleaved and activated to produce excessive mature interleukin 1β (IL-1β) and IL-18, triggering a series of immunopathogenic effects or immunopathology.[65] It was found that mitochondrial reactive oxygen species (mtROS) and lysosomal damage were necessary for PB1-F2 peptide-mediated NLRP3 inflammasome activation and immunopathology.[64] Whether and how the internalised PB1-F2

peptide perturbs lysosomal and mitochondrial function resulting in mtROS production and NLRP3 inflammasome activation remain to be elucidated. Interestingly, instead of activating NLRP3 inflammasome maturation, intracellularly expressed PB1-F2 seems to suppress NLRP3 inflammasome activation as demonstrated in NLRP3 inflammasome reconstitution experiment in non-phagocytic cell line HEK293T.[41] We have recently found that intracellular PB1-F2 suppresses inflammasome in an IAV subtype-dependent manner, with PB1-F2 of highly pathogenic IAV being a less potent suppressor than PB1-F2 of low pathogenicity IAV.[66] This suggests that the ability to activate NLRP3 inflammasome is specific to extracellular PB1-F2. It will be of great interest to clarify the relationship between the inflammasome-modulating activities of intracellular and extracellular PB1-F2. Whether the full-length and truncated PB1-F2 might also behave differently in the activation of NLRP3 inflammasome also deserves further investigation.

The ability of extracellular PB1-F2 to activate NLRP3 inflammasome is also IAV strain-specific and is conserved only in high-pathogenicity strains. Whereas PB1-F2 from H3N2 of the 1968 pandemic is highly immunopathogenic, progressively decreasing immunopathogenicity was seen in its counterparts in H3N2 pandemic descendants.[63] By sequence analysis, it was found that the "proinflammatory domain" comprising L62, R75, R79 and L82 in PB1-F2 of pandemic H3N2 was necessary for PB1-F2-mediated immunopathology, while mutations to P62, H75, Q79 and S82 in PB1-F2 of H3N2 descendants abolished the immunopathogenic effect.[63] A proinflammatory domain was also found in PB1-F2 of H1N1 of the 1918 pandemic and H2N2, but was gradually lost in all their descendants,[67] suggesting that extracellular PB1-F2-mediated immunopathology shapes the virulence of pandemic IAV, but is lost plausibly due to IAV adaption to humans.

Besides, extracellular PB1-F2 can mediate a novel type of cell death. It was found that extracellular PB1-F2 peptides with "cytotoxic domain" of I68, L69 and V70 were not only

immunogenic, but also cytotoxic to cells. On the contrary, mutations of I68T, L69Q and V70G relieve extracellular PB1-F2-mediated cell death.[68] Instead of being pro-apoptotic, extracellular PB1-F2-mediated cytotoxicity is necrotic, as demonstrated by its insensitivity to pan-caspase inhibitor,[68] and is executed through direct lysis of cell membrane.[69] Necrosis is one type of cell death response in which cellular content is released to act as proinflammatory mediators.[70] Whether extracellular PB1-F2-mediated necrosis triggers a second level of inflammatory response is still an unanswered question. Extracellular PB1-F2-mediated necrotic cell death was also a key in the promotion of secondary bacterial infection.[68] Plausibly, extracellular PB1-F2 represses anti-bacterial immunity and increased bacterial adhesion by inducing necrotic death of immune cells and lung epithelial cells.[71]

4. PB1-F2 modulation of type I IFN response

4.1 Delayed type I IFN response and IAV pathogenesis

In addition to modulating apoptosis and NLRP3 inflammasome activation, PB1-F2 is also capable of suppressing or activating type I interferon (IFN) response. Type I IFN is a major antiviral cytokine that activates multiple interferon-stimulated genes to restrict viral replication.[72] However, excessive type I IFN can result in uncontrolled inflammation that exacerbates IAV pathogenesis.[73] As mentioned earlier in section 2, PB1-F2 is an activator of NF-κB signalling, which in turn activates type I IFN expression.[59, 60] Opposite to this, PB1-F2 can also suppress type I IFN response during IAV infection.[41, 49, 74-76] Whether suppression or activation of type I IFN prevails in the context of IAV infection depends on time or infection stage. Indeed, PB1-F2 suppresses type I IFN response at early time points from 5 to 8 hours post-infection at a multiplicity of infection (MOI) of 5 in IAV-infected A549 cells when compared to cells infected with a PB1-F2-knockout IAV.[76] However, at 24 hours post-infection and an MOI of 5, PB1-F2-proficient IAV elicits a more robust type I IFN response in

A549 cells than PB1-F2-deprived virus.[60] This suggests that PB1-F2 suppresses type I IFN production during early phase of IAV infection but changes to exert an exacerbating effect on IFN response in late phase. Notably, the observed inhibitory and augmentory effects of PB1-F2 were shown to correlate with overall immunopathology induced by IAV,[59, 76] indicating that the combined pattern known as a "delayed type I IFN response" contributes to IAV pathogenesis.

In support of this model, one molecular determinant of the delayed type I IFN response induced by PB1-F2 was identified to be a specific amino acid residue S66. PB1-F2s of highly pathogenic strains including H5N1 (HK/97) and pandemic 1918 carry S66 to enhance pathogenicity in mice when compared to non-pathogenic and less pathogenic IAVs with an N66 in PB1-F2.[77] Interestingly, S66 of PB1-F2 does not change viral replication kinetics *in vitro* in MDCK cells but boosts viral titre in lung of IAV-infected mice, implying that PB1-F2 with an S66 might delay viral clearance.[77] By transcriptomic analysis, it was found that N66S mutation of PB1-F2 suppresses early type I IFN response but exacerbates late IFN response during IAV infection.[78] Plausibly, the 'delayed' pattern of type I IFN induction by PB1-F2 with an S66 boosts viral lung titre and exacerbates lung immunopathology in IAV-infected mice.[77, 78]

4.2 Mechanistic analysis of PB1-F2 modulation of RIG-I signalling

Intense research efforts have been devoted in recent years to elucidate how PB1-F2 dysregulates type I IFN production. Indirectly, as mentioned in section 2, PB1-F2 can suppress type I IFN production by macrophages through induction of macrophage apoptotic cell death.[49] Directly, increasing evidence has demonstrated that PB1-F2 targets mitochondria and adaptor proteins such as MAVS and TBK1 to modulate RIG-I signalling.

RIG-I-dependent type I IFN response serves important antiviral function in IAV infection.[79, 80] RIG-I signalling is initiated by activation of RIG-I by viral RNA. Once bound with incoming foreign

RNA species with 5’ triphosphates and double stranded region like the panhandle of IAV RNA, the CARD domain of RIG-I is released from suppression by the helicase domain.[81, 82] Following dephosphorylation, K63-ubiquitination and interaction with accessory proteins such as PACT and 14-3-3ε, activated RIG-I oligomerizes and binds to CARD domain of MAVS adaptor protein.[81, 82] Next, MAVS protein oligomerizes to form giant protein aggregate on mitochondria[83] as a platform to recruit downstream effectors TRAFs,[84] which in turn recruit IKKs and TBK1 complex to activate transcription factors IRF3, IRF7 and NF-κB, leading to stimulation of type I IFN transcription and expression.[85]

4.2.1 PB1-F2 and MAVS

PB1-F2 can impair MAVS signalosome formation in multiple manners. It was found that PB1-F2 dissipates mitochondrial membrane potential (ΔΨm), perturbs mitochondrial dynamics and sequesters MAVS protein to suppress RIG-I-dependent type I IFN production. As mentioned in section 2, PB1-F2 can permeabilize mitochondrial membrane by channel formation or recruiting mPTP complex.[18, 42] Consistently, it was found that PB1-F2 can dissipate ΔΨm, which is necessary for MAVS signalosome formation,[86] to suppress RIG-I-dependent type I IFN production.[41, 74] Besides, dissipation of ΔΨm by PB1-F2 also activates OMA-1 that cleaves OPA-1, a mitochondrial fusion protein, but enhances Drp-1 recruitment to mitochondria to augment mitochondrial fission.[41] Mitochondrial fusion is necessary for proper MAVS signalosome formation including Mtn1 recruitment and MAVS-STING interaction at mitochondria-associated endoplasmic reticulum membranes (MAM).[87, 88] Thus, PB1-F2-mediated mitochondrial fission sabotages proper MAVS signalosome formation and blocks RIG-I-dependent type I IFN production. Moreover, PB1-F2 protein can bind to the transmembrane domain of MAVS.[74] As PB1-F2 is a self-aggregating protein that forms amyloid structure as mentioned earlier,[44-47] PB1-F2-bound MAVS protein is probably

sequestered and inactivated. Importantly, it was demonstrated that N66S mutation of PB1-F2 substantiates its suppressive effect on RIG-I-dependent type I IFN production at the level of MAVS, mediated through higher binding affinity to MAVS and more pronounced dissipation of ΔΨm.[74, 75] PB1-F2-dependent suppression of RIG-I signalling correlates with IAV pathogenesis.

On the other hand, PB1-F2 can also target MAVS signalosome to activate type I IFN production.[60] Unlike its suppressive effect on MAVS mediated through elimination of IRF3 activity but not that NF-κB or AP-1,[76] PB1-F2 activates MAVS through NF-κB but not IRF3 or AP-1.[60] Indeed, as mentioned earlier in section 2, PB1-F2 can activate NF-κB signaling by binding with NDP52.[61] Interestingly, NDP52 is found to target MAVS[89-91] and its signal transducer TRAF6[92] for autophagic degradation. PB1-F2 likely sequesters and inhibits NDP52 to relieve lysosomal degradation of MAVS and TRAF6. The remaining MAVS-TRAF6 signalosome could thus propagate an excessive activation signal for NF-κB, leading to type I IFN expression. Whether PB1-F2 activation of MAVS-TRAF6-NF-κB signaling through NDP52 contributes to the late phase of "delayed type I IFN response" as mentioned above remains an open question.

4.2.2 PB1-F2 and TBK-1-DDX3 complex

In addition to targeting mitochondria and MAVS protein, a recent study has unveiled a new and strain-specific mechanism by which PB1-F2 of H1N1 of the 1918 pandemic suppresses type I IFN production by targeting DDX3 protein to proteasomal degradation.[93] PB1-F2 of the 1918 pandemic was more prone to destruction by ubiquitin proteasome system (UPS) than the counterpart in the PR8 strain.[93] Interestingly, instead of compromising its IFN-suppressing effect, the unstable 1918 PB1-F2 can more potently suppress type I IFN production than the stable PR8 PB1-F2. Mechanistically, 1918 PB1-F2 but not PR8 PB1-F2 specifically binds to DDX3, which is a substrate and coactivator of TBK1,[94] and facilitates DDX3 degradation.[93] The unstable 1918 PB1-F2

adapts DDX3 and UPS to TBK1.[93] Ectopic administration of DDX3 recombinant protein rescued lethal infection of mice with 1918 H1N1 by resupplying sufficient amount of type I IFN,[93] suggesting that 1918 PB1-F2 affects the outcome of IAV infection by targeting DDX3 for degradation.

It remains unclear whether PB1-F2-mediated DDX3 degradation applies to other pathogenic IAV. Importantly, amino acid residues T68 and P69 specific to 1918 PB1-F2 but not PR8 PB1-F2 are necessary for destabilization of 1918 PB1-F2 and high virulence of the virus. However, destabilization of PB1-F2 is also thought to be associated with attenuation of virus. In one study, ubiquitination of PB1-F2 at C-terminal lysine cluster was found to facilitate UPS degradation.[95] When C-terminal lysine residues were mutated to arginines, PB1-F2 degradation was prevented.[95] However, the same PB1-F2 mutant was found to be more potent in the inhibition of type I IFN response.[95] Another study found that residues T68, Q69, D70 and S71 destabilize PB1-F2, while mutation to their natural counterparts I68, L69, V70 and F71 enhances PB1-F2 stability.[96] Similarly, it was demonstrated that stable PB1-F2 with I68, L69, V70 and F71 is a more potent IFN suppressor.[96] Thus, further investigations are required to clarify exactly how protein stability of PB1-F2 of the 1918 and other strains might affect infection outcome. In this regard, the specific interaction between 1918 PB1-F2 and DDX3 is another molecular determinant of the pathologic IFN-suppressing effect mediated by 1918 PB1-F2. Mapping the specific regions or amino acid residues essential for the interaction of 1918 PB1-F2 with DDX3 is thus necessary to provide more mechanistic insight on PB1-F2-mediated DDX3 degradation and IFN suppression.

5. Concluding remarks and a unified model

In summary, PB1-F2 is a virulence factor that modulates host innate immunity to determine the outcome of IAV infection. It is an “immune cell killer” that induces apoptotic death of immune

cells, leading to elimination of immune cell-mediated antiviral immunity. It is also an “immune cell trap” that not only promotes cell death response, but also activates NF-κB signalling to induce proinflammatory cytokine and chemokine expression, resulting in leukocyte infiltration. PB1-F2 can also elicit pathogenic inflammation extracellularly through activation of NLRP3 inflammasome to generate excessive IL-1β and IL-18. Extracellular PB1-F2 may also trigger necrotic cell death and secondary bacterial infection. Although how PB1-F2 is released to extracellular space and how extracellular PB1-F2 enters the target cells remain mysterious, extracellular PB1-F2 is generally thought to be immunopathogenic. Besides, PB1-F2 can delay type I IFN response by suppressing type I IFN production at an early phase of infection but exacerbating it at a late phase. The delayed type I IFN response mediated by PB1-F2 promotes IAV pathogenesis. Mechanistically, PB1-F2 suppresses RIG-I-dependent type I IFN production by decomposing MAVS signalosome through (1) dissipating ΔΨm, (2) enhancing mitochondria fission but suppressing mitochondria fusion, and (3) directly binding and inhibiting MAVS protein. In addition, PB1-F2 can enhance UPS degradation of DDX3, leading to inactivation of TBK1. At later stage of IAV infection, PB1-F2 enhances RIG-I-dependent type I IFN response through activation of NF-κB signalling. PB1-F2 binds NDP52 that is an essential autophagic receptor of MAVS and TRAF6. Probably, in some occasions, PB1-F2 might outcompete MAVS and TRAF6 for NDP52 binding. As such, MAVS and TRAF6 are no longer degraded and remain active to propagate the signal for NF-κB activation and type I IFN production. Some existing knowledge on PB1-F2 is derived from single cell types, it is desirable that multiple cell types and *in vivo* models are used to verify key findings.

Here, a unified model of PB1-F2-mediated IAV pathogenesis is proposed (Figure 1). As shown on the left-hand side of the figure, at early stage of infection, PB1-F2 stays monomeric in IAV-infected epithelial cells with minimal apoptosis. In contrast, rapid oligomerization of PB1-F2 in alveolar macrophages could elicit pronounced apoptotic cell death that abolishes phagocytic

antiviral immunity and production of type I IFN. In lung epithelial cells, PB1-F2 suppresses type I IFN antiviral response by disrupting MAVS and/or TBK1-DDX3 signalosome critical to RIG-I signalling. PB1-F2 suppresses antiviral immunity in both alveolar macrophages and lung epithelial cells, facilitating IAV propagation. During later stage of infection as shown on the right-hand side of the figure, PB1-F2 interacts with NDP52 to activate NF-κB signalling and maintains MAVS-TRAF6 signalosome to promote type I IFN and proinflammatory cytokine production, thereby sustaining inflammation and immune cell infiltration. In this late stage, PB1-F2 oligomerizes in IAV-infected epithelial cells. The infiltrated immune cells phagocytose PB1-F2 aggregate. The ingested PB1-F2 aggregate promotes lysosomal damage and mtROS that exaggerates NLRP3 inflammasome maturation to produce IL-1β and IL-18, further substantiating the proinflammatory response in infected lung tissue. In addition to its direct effect on NLRP3 inflammasome, PB1-F2-mediated NF-κB signalling might also increase the expression of pro-IL-1β and NLRP3, leading to more pronounced inflammasome activity. Either from cell death or active secretion, PB1-F2 is released from the cells and extracellular PB1-F2 induces necrotic cell death, that impairs the structure of tracheal lining, skyrockets proinflammatory response and elicits secondary bacterial infection. Continuous PB1-F2-mediated necrotic cell death likely increases the level of extracellular PB1-F2 as in a possible feedback loop that amplifies the overall pathological response. The resulting high viral and bacterial titre in the lung and immunopathology contribute to IAV pathogenesis.

6. Outstanding research questions

Many questions concerning PB1-F2 and its roles in innate immunity and viral pathogenesis remain unanswered. Among these unanswered questions, the following three are of high priority.

First, in addition to apoptosis, can PB1-F2 activate other types of programmed cell death such as pyroptosis, necroptosis and ferroptosis in immune cells? One related issue concerns how

PB1-F2 might affect autophagy. It will be of great interest to see whether PB1-F2 from some types of IAV could trigger immune cell death through one of the above alternative pathways. Importantly, excessive neutrophil infiltration contributes substantially to PB1-F2-mediated IAV pathogenesis.[58, 59] NETosis is a neutrophil-specific cell death in which stressed neutrophils extrude its cellular content, namely neutrophil extracellular traps (NETs), to the extracellular space to "trap" pathogens.[97] Excessive NETosis has been suggested to play a role in IAV pathogenesis by disrupting endothelial and epithelial lining of respiratory tract.[98, 99] Recently, Gasdermin D, another direct substrate to active caspase 1,[100] has been found to mediate neutrophilic NETosis by promoting nuclear delobulation, nuclear expansion and plasma membrane permeabilization.[101, 102] It remains to be elucidated as to whether extracellular PB1-F2-mediated NLRP3 inflammasome activation, which involves activation of caspase 1 and Gasdermin D, might also enhance NETosis. It is however noteworthy that appropriate NETosis is vital to antiviral response against IAV infection.[103, 104] While PB1-F2-mediated apoptosis should limit the antiviral efficacy of NETosis, PB1-F2-mediated lytic necrosis should exacerbate the immunopathogenic effect of NETosis. It will be of great interest to see if there is a stage-dependent effect (Figure 1). In this model, intracellular PB1-F2 promotes apoptotic cell death of IAV-infected neutrophils, restricting the production of antiviral NETosis during the early stage of IAV infection. In contrast, when the infection progresses to the late stage of infection, extracellular PB1-F2 exacerbates Gasdermin D-mediated NETosis by excessive NLRP3 inflammasome activation as well as inflammatory necrosis through direct membrane lysis.

Second, how is PB1-F2 released from infected cells? There could be several possibilities for PB1-F2 release. It might be secreted through a non-canonical pathway just like the Tat protein of human immunodeficiency virus type 1.[105,106] It could also be released through exocytosis and exosome.[107-109] In connection to this, whether extracellular PB1-F2 of full length has the same inflammasome-activating property as the PB1-F2 peptides used in previous studies warrant

clarification. Importantly, it will be intriguing to determine how extracellular PB1-F2 acts on the target cells. Can it pass through the plasma membrane freely or is there a receptor? The molecular basis for the differential activity of intracellular and extracellular PB1-F2 remains to be elucidated. Is full-length PB1-F2 proteolytically modulated to switch on or off an activity? Can truncated PB1-F2 interact with full-length PB1-F2 to alter its activity?

Finally, what is the molecular mechanism by which the IFN-modulating activity of PB1-F2 is regulated? Plausibly, different pathways and targets might be modulated by PB1-F2 at different stages of infection. Elucidation of these and other questions surrounding PB1-F2 and innate immunity might derive new knowledge and strategies for prevention and control of IAV.

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Figure legend:

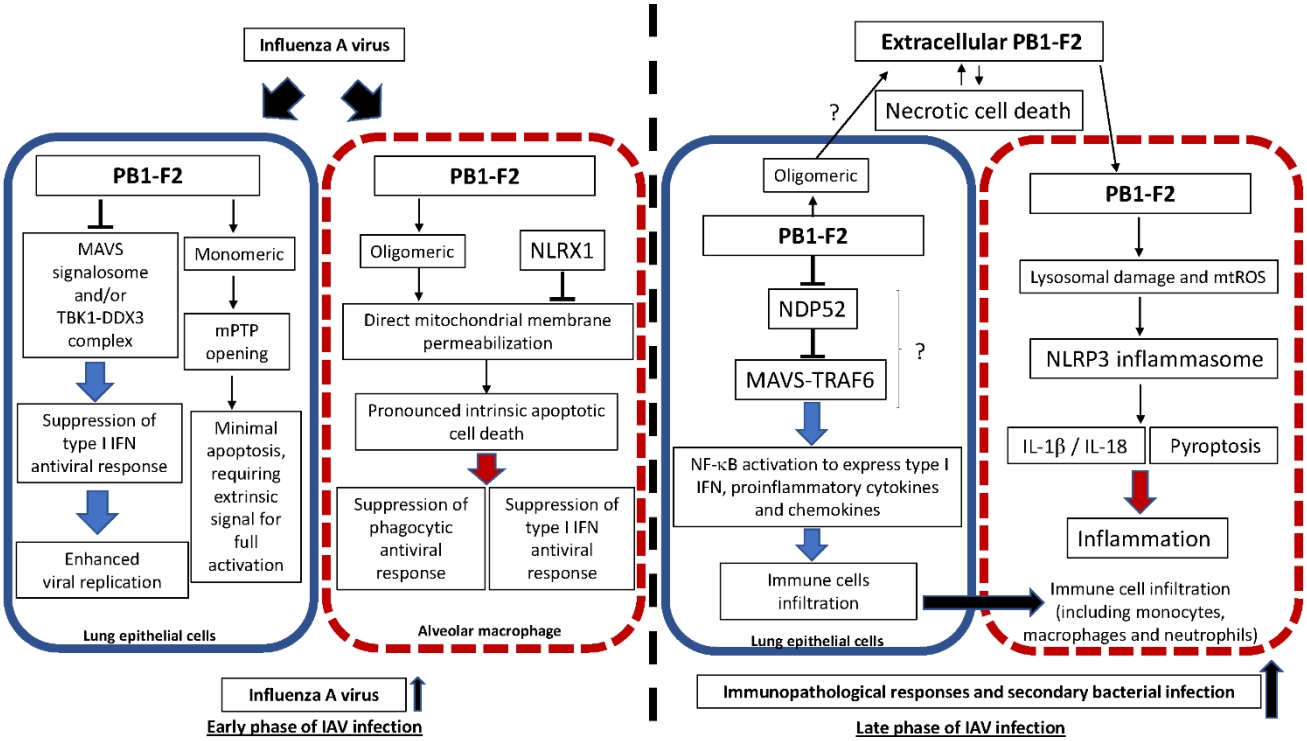
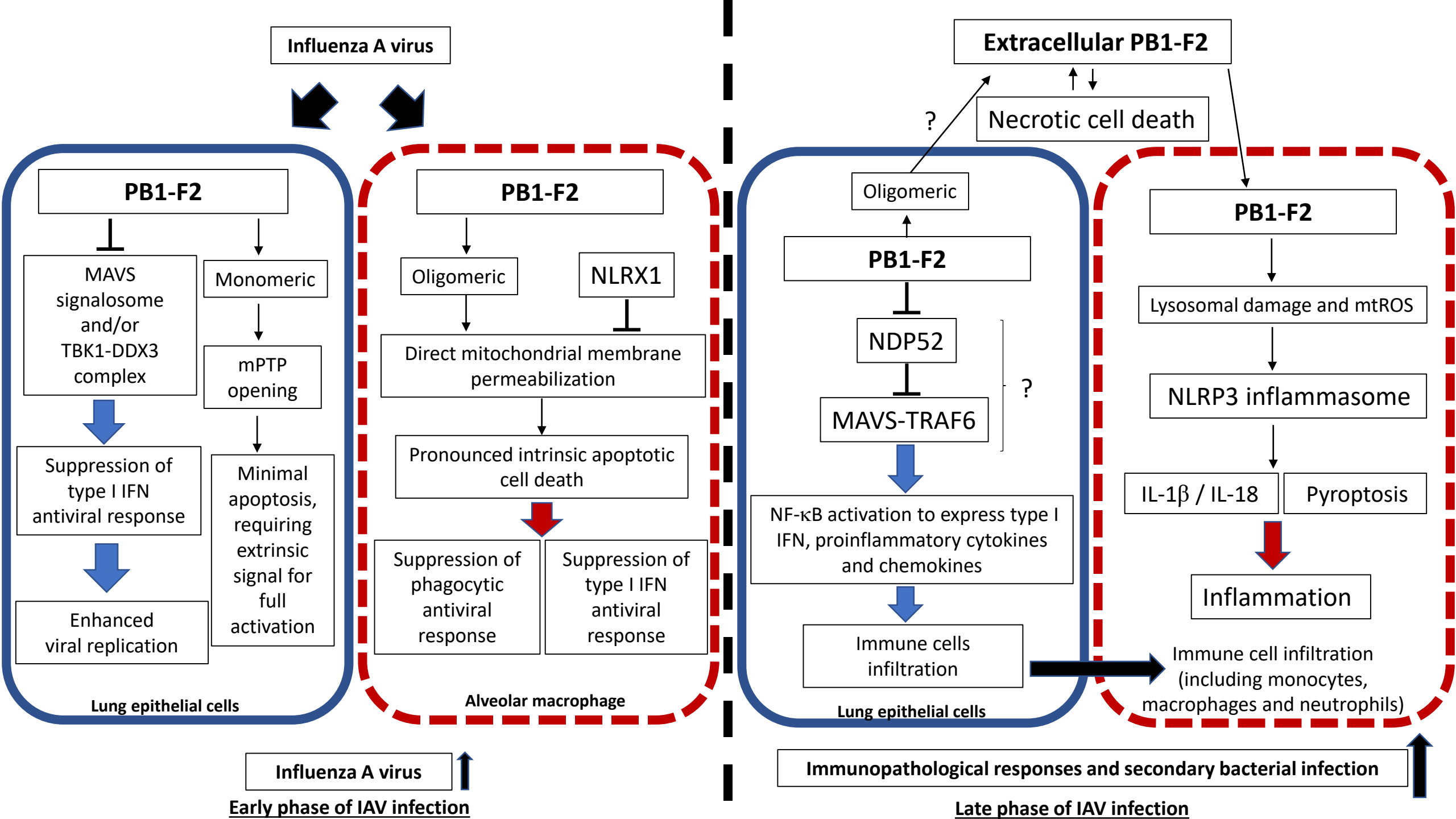


FIGURE 1 An overall model of PB1-F2-mediated immunopathology during IAV infection.


Influenza A virus
PB1-F2
MAVS signalosome and/or TBK1-DDX3 complex
Monomeric
mPTP opening
Suppression of type I IFN antiviral response
Minimal apoptosis, requiring extrinsic signal for full activation
Enhanced viral replication
Lung epithelial cells
PB1-F2
Oligomeric
NLRX1
Direct mitochondrial membrane permeabilization
Pronounced intrinsic apoptotic cell death
Suppression of phagocytic antiviral response
Suppression of type I IFN antiviral response
Alveolar macrophage
Influenza A virus
Early phase of IAV infection
Extracellular PB1-F2
?
Necrotic cell death
Oligomeric
PB1-F2
NDP52
MAVS-TRAF6
?
NF-κB activation to express type I IFN, proinflammatory cytokines and chemokines
Immune cells infiltration
Lung epithelial cells
PB1-F2
Lysosomal damage and mtROS
NLRP3 inflammasome
IL-1β / IL-18
Pyroptosis
Inflammation
Immune cell infiltration (including monocytes, macrophages and neutrophils)
Immunopathological responses and secondary bacterial infection
Late phase of IAV infection