HOST INNATE IMMUNE RESPONSES TO INFECTION BY AVIAN- AND BAT-BORNE VIRUSES

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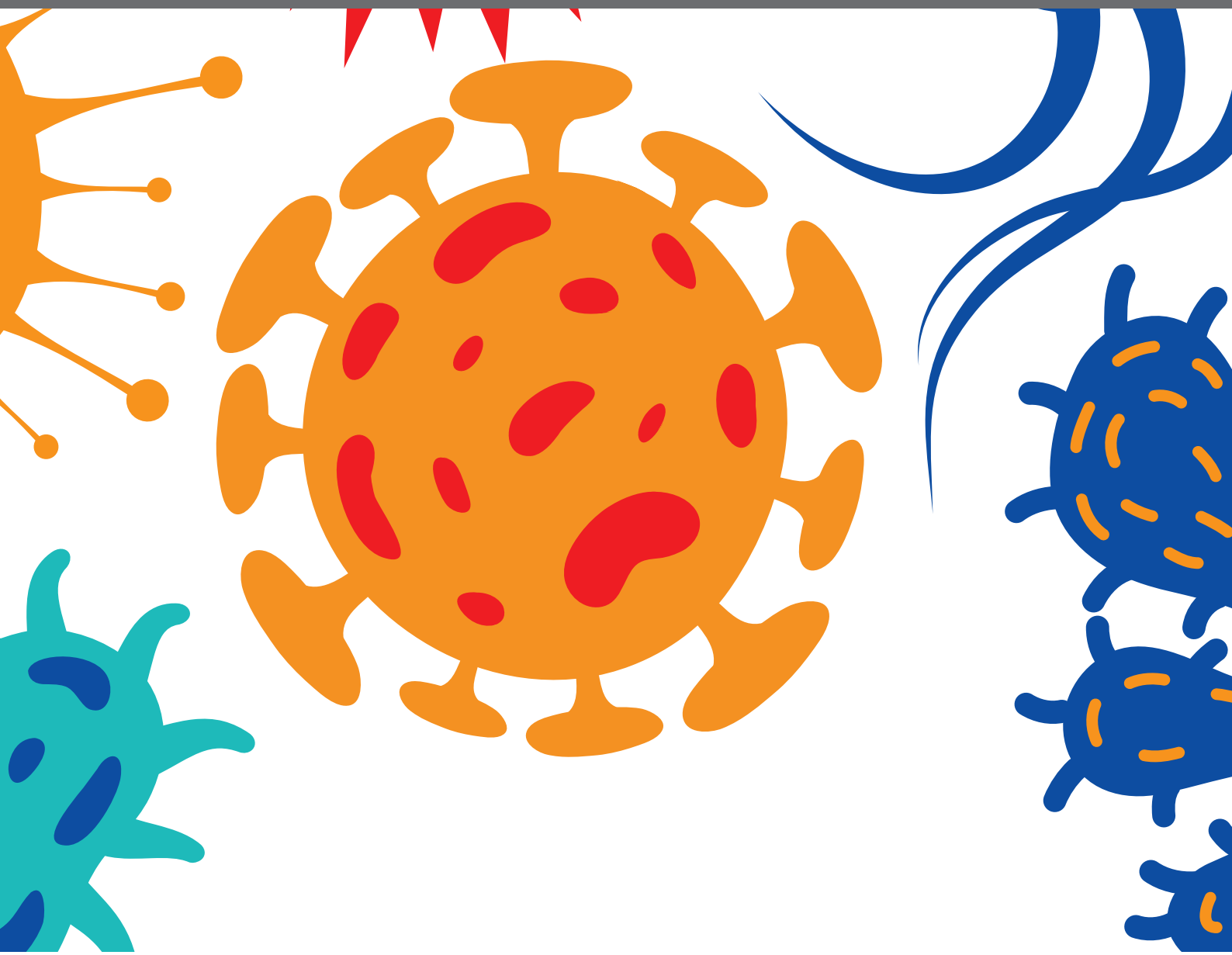

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HOST INNATE IMMUNE RESPONSES TO INFECTION BY AVIAN- AND BAT-BORNE VIRUSES

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Table of Contents

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EDITORIAL
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Editorial: Host Innate Immune Responses to Infection by Avian- and Bat-Borne Viruses

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Editorial on the Research Topic

Host Innate Immune Responses to Infection by Avian- and Bat-Borne Viruses

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The COVID-19 pandemic has generated many urgent questions on the origin, trajectory, and host preference of its causative betacoronavirus SARS-CoV-2, as well as renewed focus on other potentially zoonotic viruses. Several species of birds and wild bats can serve as reservoirs and/or mechanical vectors for many infectious viruses including influenza-A, SARS-CoV, MERS, and Ebola. Although substantial progress has been made, there are still major gaps in understanding the emergence, transmission, and adaptation of zoonotic avian- and bat-borne viruses. A major challenge is the dearth of suitable infection and immunity models. Extrapolating data from infection studies in human cell lines or rodents is limiting, as evolutionarily optimized immune factors function differently in non-hosts.

Viral infection triggers anti-viral host defenses and an inflammatory response, broadly coordinated by type I interferon (IFN) and NF-κB, respectively. IFN induces production of hundreds of "interferon-stimulated genes" (ISGs), limiting virus replication until the adaptive immune response can "clean-up." Rapid initiation of innate immune responses depends on viral recognition by pattern recognition receptors (PRRs) which are expressed in tissues where the virus replicates. Despite the high degree of evolutionary conservation, and assumed similarity in function, there are significant differences between the immune gene repertoires of birds and mammals and bats and other mammals. Ducks utilize many of the mammalian PRRs (i.e. RIG-I, TLR7, and TLR3) and their downstream adaptors to detect and inhibit the replication of influenza viruses (Campbell and Magor). In chickens, key innate immune genes, such as *IRF3* and *RIG-I*, appear to have been lost (Chen et al., 2013). However, chicken cells are capable of initiating an interferon response after virus infection and inducing the expression of hundreds of ISGs (Giotis et al., 2016; Giotis et al., 2017).

Differences in IFN-inducing capacity between virus strains is an important parameter contributing to the host response to viral infection and virulence. Influenza-A virus circulates in multiple hosts causing economic burden in the poultry industry, human epidemics, and

occasionally pandemics. Influenza-A virus contains eight negative-strand RNA segments, which are thought to encode 10 viral proteins as well as seven newly identified proteins such as PB1-F2, PA-X whose role is not entirely clear (Ma et al.). The full-length PA-X and PB1-F2 proteins in a recombinant 2009 pH1N1 virus have been reported to enhance viral replication *in vitro*, enhancing viral virulence *in vivo* mainly through simultaneously mediated host innate immune response (Ma et al.).

IBDV (causing Infectious Bursal Disease) is another economically important (but not zoonotic) virus for the poultry industry. Very virulent strains of IBDV (vvIBDV) emerged in the 1980s, causing up to 60% mortality in some commercial flocks for reasons that are poorly understood (Dulwich et al., 2017). Dulwich et al. extended these observations by demonstrating that a vvIBDV strain is able to down-regulate type I IFN and pro-inflammatory cytokine responses compared to a classical field strain both *in vitro* and *in vivo*.

The successful control of virus infection requires the coordination of both the innate and adaptive immune systems. During chronic viral infections the increased level or duration of stimulation of virus-specific CD8 T-cells leads to non-functional state called T-cell exhaustion (Freeman et al., 2006). Recent studies have shown that targeting the immunoreceptor molecule PD1 and its ligand PD-L1 can reverse the exhausted T-cell response (Freeman et al., 2006). The chicken PD-1/PD-L1 might also be used in the treatment of oncogenic avian viruses such as Marek's disease virus (Reddy et al.).

Current knowledge on bat IFN responses is rudimentary, with most of this obtained in studies of the Australian flying fox (*Pteropus alecto*) (Clayton and Munir). Sequencing of bat genomes suggests that evolution of "metabolically costly" flight imposed adaptations on their innate immune-, DNA damage- and inflammatory-response systems (Zhang et al., 2013). Key antiviral immunity components are conserved in bats, *e.g.* IFNs and their receptors and ISGs However, genes that activate the inflammasome and/or IFN pathways are either missing or have altered function (Clayton and Munir). Studies have inferred a pattern of constitutively expressed IFN-α in unstimulated *Pteropus alecto* cells, which may provide a "switched-on" defense mechanism that blunts virus replication and pathogenesis (Zhou et al., 2016), and can explain why bats are asymptomatic reservoirs of viruses. The remarkable ability of bats to coexist with a wide range of potentially zoonotic viruses is exemplified by the evolutionarily distinct bat influenza-A-like viruses H17N10 and H18N11 (BatIVs). Unlike classical influenza-A viruses, the surface glycoproteins of BatIVs neither bind nor cleave sialic acid receptors, but rather use the trans-species conserved MHC-II proteins (Giotis et al., 2019) to gain cell entry. The scientific evidence so far indicate a limited spillover risk for BatIVs, but data is not conclusive enough to dismiss the possibility of zoonotic transmission (Giotis).

Full-length genome sequences obtained from COVID-19 patients demonstrated that SARS-CoV-2 shares 79.5% sequence identity to SARS-CoV BJ01 strain and a 96% sequence identity to the bat coronavirus RaTG13, suggestive that bats are the original source of the virus (Zhou et al., 2020). The receptor binding protein spike (S) viral gene is highly divergent to other CoVs but has a 93.1% nucleotide identity to RaTG13 (Zhou et al., 2020). Molecular modeling and functional studies revealed that the host surface protein ACE2 mediates cell entry of SARS-CoV-2 and the serine protease TMPRSS2 is essential for S priming (Minakshi et al.). Furthermore, the aftermath of SARS-CoV-2 internalisation is governed by a complex and largely unknown network of host-pathogen interactions. Transcriptomic analysis of human alveolar and bronchial epithelial cells confirmed that the *CSF1* gene, a known target of the microRNA miR-1207-5p, is over-expressed following SARS-CoV-2 infection (Bertolazzi et al.). CSF1 enhances macrophage recruitment and activation and its overexpression may contribute to the acute inflammatory response observed in severe COVID-19.

In conclusion, recent virus outbreaks confirmed the inextricable nature of human and animal health and disease. Interdisciplinary approaches are urgently needed to assess and prevent the spillover of avian- and bat-borne viruses using an organismal biology approach and focusing on viral ecology, diversity, and interactions with the host.

AUTHOR CONTRIBUTIONS

EG wrote the first draft and JS and DM edited and commented on the draft. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Effects of the PA-X and PB1-F2 Proteins on the Virulence of the 2009 Pandemic H1N1 Influenza A Virus in Mice

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There have been several previous reports showing that PA-X and PB1-F2 proteins can regulate innate immune responses and may play roles in the adaptation of influenza viruses to new hosts. In this research, we investigated, for the first time, the combined effects of PA-X and PB1-F2 proteins on viral virulence in mice. Based on the 2009 pH1N1 A/Guangdong/1057/2010 virus backbone, four viruses encoding different combinations of full-length or truncated PA-X and PB1-F2 proteins were rescued by a reverse genetic engineering system. We analyzed viral replication, host-shutoff activity, *in vitro* viral pathogenicity and *in vivo* host immune response. We found that simultaneously expressing the full-length PA-X and PB1-F2 proteins enhanced viral replication *in vitro* through increasing the accumulation of the RNP complex protein and enhanced viral pathogenicity in mice during the early stage of infection. Furthermore, PA-X and PB1-F2 simultaneously regulated the host innate response, and different forms of PB1-F2 proteins may have impacts on the host shutoff activity induced by the PA-X protein. Our results provide a better understanding of the mechanisms of PA-X and PB1-F2 proteins during viral replication, pathogenicity and host immune response.

Keywords: influenza, PA-X, PB1-F2, mice, host-shutoff

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INTRODUCTION

Influenza A virus circulates in multiple hosts ranging from aquatic or terricolous birds to diverse mammalian species, including humans. Influenza A virus contains eight single negative-strand RNA segments, which are thought to encode 10 viral proteins (PB2, PB1, PA, NP, NA, M1, M2, NS1, and NS2) (Lamb and Lai, 1980; Lamb et al., 1981). However, in the past few years, seven new proteins encoded by the virus genome have been identified, including PB1-F2 (Chen et al., 2001), PB1-N40 (Wise et al., 2009), PA-X (Jagger et al., 2012), M42 (Wise et al., 2012), NS3 (Selman et al., 2012), PA-N155, and PA-N182 (Muramoto et al., 2013). Among these novel discovered proteins, PA-X and PB1-F2 proteins have been discovered to have functions in regulating the host immune response and are suspected to play roles in the adaptation of influenza to mammalian hosts.

The PA-X protein, as a result of a +1 ribosomal frameshift, undergoes a change at a specific location in the PA mRNA that results in a change in activity, shares the same N-terminal 191 aa sequence with the corresponding PA protein, and has a unique C-terminal 41 aa (truncated) or

61 aa (full-length) sequence encoded by an overlapping open-reading frame (ORF) (Jagger et al., 2012). PA-X exhibits endonuclease activity though the same N-terminal amino acid sequence as the corresponding PA protein, and the C-terminal part of PA-X contributes to the suppression of host protein synthesis (Hayashi et al., 2015; Xu et al., 2016). Furthermore, PA-X can selectively degrade mRNAs transcripts produced by the host RNA polymerase II, but it ignores the activity of RNA polymerase I and III (Khaperskyy et al., 2016).

The multiple effects induced by the PA-X protein have been studied *in vitro* and *in vivo*. The PA-X protein can modulate the host immune response (Gao et al., 2015b) and has effects on viral replication and pathogenicity (Lee et al., 2017). In addition, phylogenetic analysis of over 3,000 PA gene sequences demonstrated that the truncated PA-X protein overwhelmingly comes from mammalian influenza, indicating that the PA-X protein may be associated with the adaptation of IAV from avian hosts to mammalian species (Shi et al., 2012).

The PB1-F2 protein is encoded by a +1 ORF in the PB1 sequence as a result of leaky ribosomal scanning, which is likely triggered by an optimal Kozak sequence at the start codon of PB1-F2 (Chen et al., 2001). Several functions of the PB1-F2 protein have been described, such as inducing cell death (Chen et al., 2001), host innate immune response regulation (Leymarie et al., 2013), and viral polymerase activity enhancement (Mazur et al., 2008). However, the effects caused by PB1-F2 in regard to virulence were cell type, strain, and host specific. Therefore, the PB1-F2 protein is known as a crucial virulence factor of IAV but the common mechanisms inducing the virulence change are still unclear. There are multiple versions of the PB1-F2 protein with different lengths, but generally avian-original PB1-F2 proteins consist of the full-length 90 aa and a truncated 11 aa version comes from human and swine influenza. Thus, several studies have suggested that truncated PB1-F2 proteins contribute to the adaptation of the virus to mammalian hosts (Alymova et al., 2014; Kamal et al., 2017).

Since the 2009 pandemic, when the H1N1 (Pdm09) influenza virus was discovered in North America and spread throughout the entire world, H1N1 has caused severe economic losses and remains a significant threat to public health. The Pdm09 viruses express both the truncated PB1-F2 (11 aa) and PA-X (232 aa) proteins, and the functions of these proteins have been well-characterized. Lee et al. (2017) found that the expression of PA-X increased viral virulence *in vitro* and *in vivo* when compared with a deficiency virus, Cal/09. For the PB1-F2 proteins, studies have found that restoring the truncated PB1-F2 protein of the Pdm09 virus has minimal effect on viral pathogenicity in mice and pigs (Hai et al., 2010; Pena et al., 2012). However, although some studies indicated the similarity of the PA-X and PB1-F2 proteins, such as in promoting mammalian adaptation and regulating the host immune response, the combined effects of the PB1-F2 and PA-X proteins on viral virulence as well as the host immune response are still unclear.

In this study, we used the 2009 pH1N1 A/Guangdong/1057/2010(GD1057) virus as a backbone and used a genetic reverse engineering system to generate four reconstructed viruses to investigate the comprehensive effects of the four types of expression forms of the PA-X and PB1-F2 proteins (truncated PA-X and PB1-F2, truncated PA-X with full-length PB1-F2, full-length PA-X with truncated PB1-F2, full-length PA-X and PB1-F2) on viral replication, pathogenicity and host innate response.

MATERIALS AND METHODS

Ethics Statement

All animal studies were conducted under the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of the People's Republic of China and were approved by the animal experimental ethics committee of the South China Agricultural University (approval number 2013-07).

Cells and Viruses

Madin-Darby canine kidney (MDCK), human embryonic kidney cells (293T), and porcine kidney (PK-15) cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) complemented with 10% fetal bovine serum (FBS; Gibco, USA) and 1% penicillin-streptomycin (HyClone, USA) at 37°C and 5% CO_2.

A/Guangdong/1057/2010 (GD1057) is a Pdm09 H1N1 influenza virus strain that was isolated in 2010 from human with severe respiratory symptoms in Guangdong, China (**Table S1**), and preserved in our laboratory. Virus stocks were propagated in MDCK cells for three passages and sequenced before use.

Generation of Viruses by Reverse Genetic Engineering

All eight segments from GD1057 were amplified by RT-PCR (**Table S2**) and cloned into plasmid PHW2000 as described previously (Hoffmann et al., 2000), which were named WT_X. To rescue viruses expressing the full-length PA-X protein, a single codon mutation was introduced from UAG (stop) to UGG (tryptophan) at position 42 in the C-terminal domain of the PA-X protein based on WT_PA. The plasmid was named Pdm09_PAX_61. Three nucleotide substitutions (153A to C, 291A to G, and 381A to G) were introduced into the WT _PB1 plasmid to prolong the coding PB1-F2 protein from 11 to 90 aa (**Table S3**), and the resulting plasmid was named Pdm09_F2_90. None of the mutations in the plasmids changed PA or PB1 ORF production. Mutations were introduced with the Fast-directed Site-directed Mutagenesis kit (TIANGEN, China) by following the manufacturer's instructions, and every plasmid was confirmed by sequencing.

To analyze the effects of the PA-X and PB1-F2 proteins in GD1057, wild type (WT_PAX_41/F2_11) and three mutated recombinant viruses (Pdm09_PAX_41/F2_90, Pdm09_PAX_61/F2_11, Pdm09_PAX_61/F2_90) were rescued by the PHW2000 eight-plasmid system as described previously (Hoffmann et al., 2000). Briefly, a monolayer of 293T cells with ∼90% confluence in six-well plates were cotransfected with eight constructed PHW2000 plasmids (WT_PB2, WT_PB1 or Pdm09_F2_90, WT_PA or Pdm09_PAX_61, WT_HA, WT_NP, WT_NA, WT_M, and WT_NS) encoding viral genomic RNA

segments using Lipofectamine™ 3000 Transfection Reagent (Invitrogen). Then, 24 h of incubation, the supernatant of the 293T cell culture was harvested and passaged three times in MDCK cells to prepare the viral stocks. The rescued viruses were confirmed by virus genome sequencing.

Viral Titration and Replication Kinetics

Before using the stock of viruses, $TCID_{50}$ of each virus was inoculated in MDCK cells using a 10-fold serially diluted virus inoculated at 37° C and harvested after 48 h. The $TCID_{50}$ value was calculated by the Reed and Muench method as previous studies (Xu et al., 2016; Lee et al., 2017). The experiments were done in triplicates. To evaluate the replication kinetics of the viruses, MDCK and PK-15 cells were infected with viruses at a multiplicity of infection (MOI) of 0.01. Supernatants of the infected cells were collected and stocked at 12, 24, 36, and 48 hpi. The virus titer was determined based on the $TCID_{50}$ in the MDCK cells from the $TCID_{50}$, and three independent repeat experiments were conducted to exclude any error.

Viral RNP Polymerase Activity Assay

To evaluate the effects of the PA-X and PB1-F2 proteins on the polymerase activity, a ribonucleoprotein (RNP) mini-genome assay was conducted as described previously (Tan et al., 2014). Briefly, 293T cells in 12-well plates were cotransfected with 0.25 μg of each RNP complex expression plasmids (WT_PB2, WT_PB1 or Pdm09_F2_90, WT_PA or Pdm09_PAX_61, WT_NP), the luciferase reporter plasmid pPolI- Luci -NP and a 0.025 μg Renilla luciferase expressing plasmid as an internal control by using Lipofectamine 3000 (Invitrogen, USA), as recommended by the manufacturer. Cells transfected without the PA plasmid were used as a negative control. Then, 24 h post-transfection, cells in the wells were harvested, and luciferase signals were determined with a Dual-Luciferase Reporter Assay System (Promega, USA).

Western Blot Analysis

Total cell protein lysates were extracted from the transfected 293T cells with CA630 lysis buffer (150 mM NaCl, 1% CA630 detergent, 50 mM Tris base [pH 8.0]). Proteins were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Merck Millipore, USA). Each PVDF membrane was blocked with 0.1% Tween 20 and 5% non-fat dry milk in Tris-buffered saline and subsequently incubated with a primary antibody. The primary antibodies were specific for influenza A virus PA (1:3,000, GeneTex, USA), influenza A virus PB1 (diluted 1:3,000, GeneTex, USA), influenza A virus NP (1:3,000, GeneTex, USA), and influenza A virus PA-X (diluted 1:2,000, polyclonal rabbit antiserum against the PA-X derived peptide (CAGLPTKVSHRTSPA) (diluted 1:3,000 Genscript, China). The secondary antibody used was either horseradish peroxidase (HRP)-conjugated anti-mouse antibody or HRP-conjugated anti-rabbit antibody (diluted 1:10,000 Beyotime USA) as appropriate. HRP presence was detected using a Western Lightning chemiluminescence kit (Amersham Pharmacia, Freiburg, Germany), following the manufacturer's protocol.

GFP Expression Assay

293T cells in 6-wells plates were cotransfected with 400 ng of pEGFP-N1 plasmid (Clontech), 400 ng of PA (PAX_41 or PAX_61) plasmid and 400 ng of PB1 (PB1_F2_11 or PB1_F2_90) plasmid. 293T cells were cotransfected with 400 ng of pEGFP-N1, and 800 ng of empty PHW2000 was used for the control group. Then, 24 h post-transfection, the cells were harvested, and their mean fluorescent intensity was measured using flow cytometry (Beckman Coulter, USA) and FlowJo (treestar) software. The fluorescence intensity of cells was calculated after normalizing to the control group (taken as 100%). Three independent experiments were performed. Cell lysates from each group were used to evaluate the expression levels of PB1, PA, and GFP (1:1000, GeneTex, USA) by Western blotting. β-actin was used as a loading control. Detected protein bands were quantified using densitometry (Image-Pro Plus, Media Cybernetics) and the expression levels of PB1, PA, and GFP were normalized to β-actin.

Mouse Infections

A total of 80 6-week-old female BALB/c mice (Guangdong Medical Laboratory Animal Center, China) were randomly divided into five groups (14 mice/group). Mice from each group were slightly anesthetized with dry ice and inoculated intranasally (IN) with 50 μL DMEM fluid containing 10^6 $TCID_{50}$ of each virus or 50 μL of virus-free DMEM. Mice were monitored daily for weight loss and clinical signs until 14 days post-inoculation. If a mouse had decreased in body weight of more than 25% compared with their original body weight, they were considered dead and were euthanized. At day 1, 3 and 5 days post-infection, three randomly selected mice in each group were euthanized, and their lungs were collected and stored in order to analyze virus titers, the host innate immune response and to conduct histopathology assays. To determine the virus titer in the lungs, 10% lung homogenates were made in DMEM with 1% antibiotic supplementation. Virus titers in the lung homogenates were determined by $TCID_{50}$ assays on MDCK cells as mentioned above.

Histopathology

At 5 days post-infection, lung tissues of euthanized mice were collected and immersed in 10% phosphate-buffered formalin then embedded in paraffin 48 h later. After that, samples from the same part of the lungs were cut into 5-μm-thick sections and one section from each sample was stained by hematoxylin and eosin (H&E).

RT-qPCR

Total RNA in the lung homogenates was extracted by using TRIzol® Reagent RNA (Invitrogen) following the manufacturer's protocol. Total RNA from each sample was extracted and purified prior to cDNA synthesis completed by using a PrimeScriptTMII 1st Strand cDNA Synthesis Kit (Takara) with random primers. The mRNA expression level of cytokines/chemokines, including

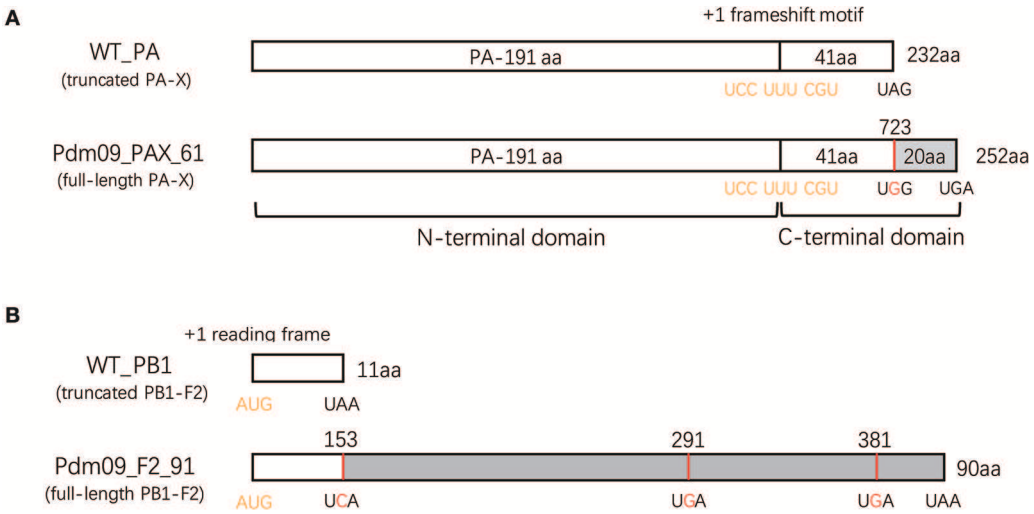

FIGURE 1 | Generation of reassortment viruses expressing full-length or truncated PA-X and PB1-F2 proteins. **(A)** Schematic diagram of the PA sequence expressing the PA-X proteins. A conserved motif in the 592 bp of the N-terminal PA sequence may cause +1 ribosomal frameshifting (indicated in yellow) when expressing PA proteins. Consequently, this frameshift produces a PA-X protein that contains the same N-terminal 191 aa as the PA protein and a unique 41 or 61 aa C-terminal domain. To rescue mutated viruses expressing the full-length PA-X protein, a single mutation (UAG to UGG, indicated with red) was induced at the stop codon to prolong the C-terminal PA-X protein from 41 to 61 aa. **(B)** Schematic representation of the PB1 sequence expressing PB1-F2 proteins. The PB1-F2 protein was translated by a +1 alternate ORF (indicated in yellow) in the PB1 sequence. A virus encoding the full-length PB1-F2 protein was constructed by introducing three mutations (A153C, A 291G, and A 381G, highlighted with red) in the PB1 plasmid.

TNF-α, IL-6, NLRP3, caspase-1, IL-1β, and IL-18 (**Table S4**), were quantitated by real-time PCR assays (qRT-PCR). The qRT-PCR mixture for each sample contained 10 μL 2 × TB green Premix DimerEraser (Takara), 6 μL of RNase-free H_2O, 1 μL of each primer and 2 μL of the cDNA template. The amplification program was as follows: 1 cycle of 95°C for 30 s; 40 cycles of 95°C for 5 s, 55°C for 30 s, and 72°C for 30 s; and finally, 1 cycle of 95°C for 5 s, 60°C for 1 min and 95°C for 5 s. Each cDNA template was tested with three experimental replicates to guarantee the quality of the data, and the results were calculated by the $2^{-\Delta\Delta Ct}$ method after normalization to the GAPHD gene expression level as an internal control.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism software version 5.00 (GraphPad Software Inc., San Diego, CA). Comparisons between two treatment means were conducted using two-tailed Student's *t*-test, whereas multiple comparisons were carried out by two-way analysis of variance (ANOVA) considering time and virus as factors. The differences were considered statistically significant at P-value < 0.05.

RESULTS

Generation of Pdm09 H1N1 Viruses Expressing the Indicated Full-Length or Truncated PA-X and PB1-F2 Proteins

To evaluate the effects of the PA-X and PB1-F2 proteins on viral replicability, pathogenicity and host innate immune response, four viruses based on the GD1057 backbone were rescued after considering various combinations of truncated or full-length PA-X and PB1-F2 proteins. Wild type (WT_PAX_41/F2_11) virus expressed truncated 232 aa PA-X and 11 aa PB1-F2 proteins. The Pdm09_PAX_41/F2_90 virus was constructed by introducing three mutations into stop codons (153A to C, 291A to G and 381A to G) in the PB1 sequence, which consequently expressed truncated 232 aa PA-X and full-length 90 aa PB1-F2 proteins (**Figure 1B**). Pdm09_PAX_61/F2_11 was rescued after substituting the stop codon with tryptophan at the 42nd amino acid position of X-ORF in the PA sequence, and it expressed the full-length 252 aa PA-X protein and truncated 11 aa PB1-F2 protein (**Figure 1A**). Pdm09_PAX_61/F2_90 was rescued with the constructed plasmids and expressed full-length 252 aa PA-X and 90 aa PB1-F2 proteins. The PA-X protein could be detected in 293T cells from all four virus transfection groups (**Figure S1**), but we failed to detect PB1-F2 with a polyclonal antibody by western blotting.

Full-Length PA-X and PB1-F2 Increase Virus Early Replication *in vitro* via Improving the Expression of Polymerase Components

To characterize all four reconstructed viruses *in vitro*, we inoculated each virus into MDCK and PK-15 cells with MOI 0.01 (**Tables S5**, **S6**). Both Pdm09_PAX_61/F2_11 and Pdm09_PAX_41/F2_90 viruses replicated slower in the MDCK cells and faster in the PK-15 cells at 24 and 36 hpi compared with the WT_PAX_41/F2_11. Interestingly, Pdm09_PAX_61/F2_90 grew to a high titer ($p < 0.05$) significantly faster than the

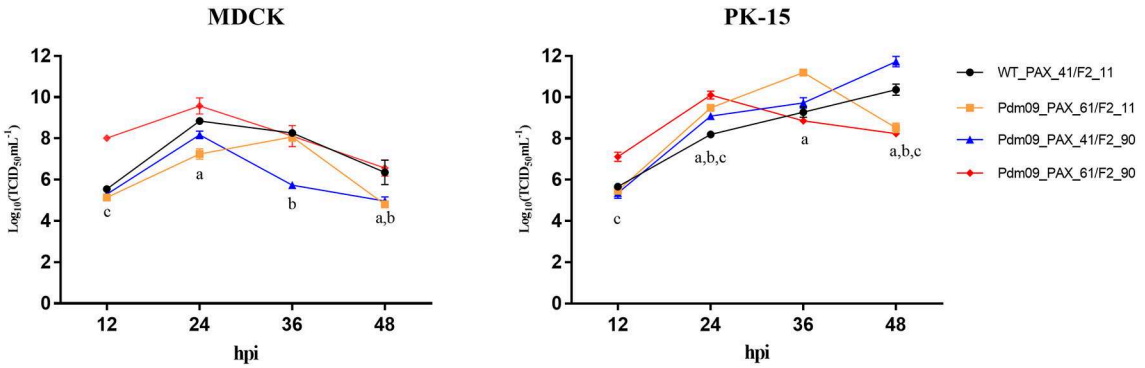

FIGURE 2 | Viral growth kinetics of rescue viruses in MDCK and PK-15 cells. MDCK and PK-15 cells were inoculated with rescue viruses at a MOI of 0.01. The supernatants were harvested at the indicated time points until 48 hpi. Each point on the lines indicates the mean ± SD of three independent experiments and significant differences ($p < 0.05$) between infected groups are marked by a, b, and c (a: WT_PAX-41/F2-11 and Pdm09_PAX_61/F2_11; b: WT_PAX_41/F2_11 and Pdm09_PAX_41/F2_90; c: WT_PAX_41/F2_11 and Pdm09_PAX_61/F2_90).

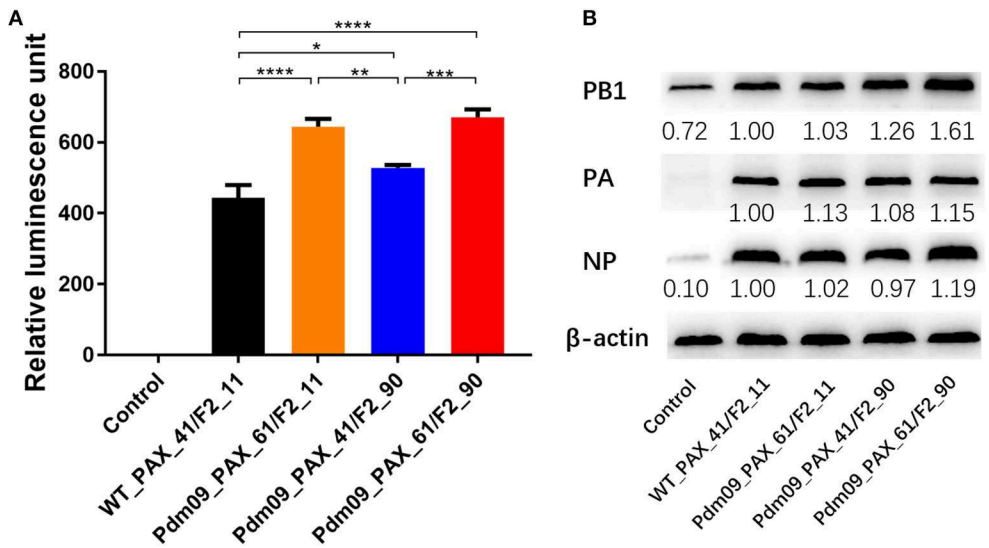

FIGURE 3 | Effects of different expression of PA-X and PB1-F2 proteins on polymerase activity and expression of RNP components. **(A)** Comparison of polymerase activities of vRNPs with the indicated different expression of PA-X and PB1-F2 proteins in 293T cells. Relative luminescence units are presented as the mean ± SD from three independent experiments. **(B)** Expression of RNP components from 293T cells 24 h post-transfection with different combinations of individual RNP plasmids. The expression level of the RNP component relative to the expression of the control, β-actin, was determined by densitometry using BandScan software, version 5.0. Data are representative of the mean of three independent experiments and were standardized to those for WT_PAX_41/F2_11 (taken as 100%).

other three viruses before 24 hpi in both cell lines (**Tables S7**, **S8**); however, the high titer of Pdm09_PAX_61/F2_90 could not be maintained after 24 hpi (**Figure 2**). The data demonstrated that the simultaneous expression of PA-X and PB1-F2 proteins enhanced virus replication in the early infection stage *in vitro*.

To measure whether the changes induced by the PA-X and PB1-F2 proteins were mediated via impacts on the polymerase activity, a dual-luciferase reporter assay was conducted to evaluate the viral polymerase activity. The results indicated that RNPs from WT_PAX_41/F2_11 and Pdm09_PAX_41/F2_90 showed significantly lower ($p < 0.05$) polymerase activity when compared with RNPs from Pdm09_PAX_61/F2_11 and Pdm09_PAX_61/F2_90 (**Figure 3**). This decreased activity indicated that the expression of the full-length PA-X protein could increase the polymerase activity compared with the truncated PA-X. Furthermore, plasmids encoding the full-length PB1-F2 protein slightly enhanced the polymerase activity when compared with the truncated one. Moreover, the polymerase complex from Pdm09_PAX_61/F2_90 harbored the strongest polymerase activity (**Figure 3**).

To further investigate the effects of different expression of PA-X and PB1-F2 proteins on viral RNA polymerase activity, western blot assays were conducted to determine the expression level of

RNP component proteins in 293T cells 24 h post-transfection. Our results showed that viruses with the full-length PA-X protein resulted in a higher ($p < 0.0001$) PA expression level compared with viruses with the truncated PA-X. Furthermore, viruses with the full-length PB1-F2 protein could accumulate more PB1 protein ($p < 0.001$) than the truncated PB1-F2 version. In addition, viruses simultaneously expressing full-length PA-X and PB1-F2 could accumulate PB1 and NP proteins significantly faster ($p < 0.0001$) than other three groups of viruses (**Figure 3**). In summary, simultaneous expression of full-length PA-X and PB1-F2 proteins in GD1057 could enhance viral replication in the early infection period; expression of the full-length proteins only slightly increased viral RNA polymerase activity but significantly increased the expression level of viral RNP components.

Different Forms of the PB1-F2 Protein May Induce Effects on the Host Shut-Off Activity of the PA-X Protein

Several studies have revealed that PA-X protein contributes to the global host shut-off activity of influenza virus. To measure whether PB1-F2 proteins have impacts on the shutoff activity of PA-X protein, 293T cells were cotransfected with plasmids expressing GFP, individual plasmids expressing PA (expressing full-length or truncated PA-X) and corresponding plasmids expressing PB1 (expressing full-length or truncated PB1-F2) for 24 h. Relative GFP expression was determined through the assessment of fluorescent intensity (**Figure S2**) and western blot assays. GFP expression of PAX_61+PB1_F2_90 was significant lower ($p < 0.05$) than PAX_41+PB1_F2_11 and PAX_41+PB1_F2_90. PAX_61+PB1_F2_11 values were

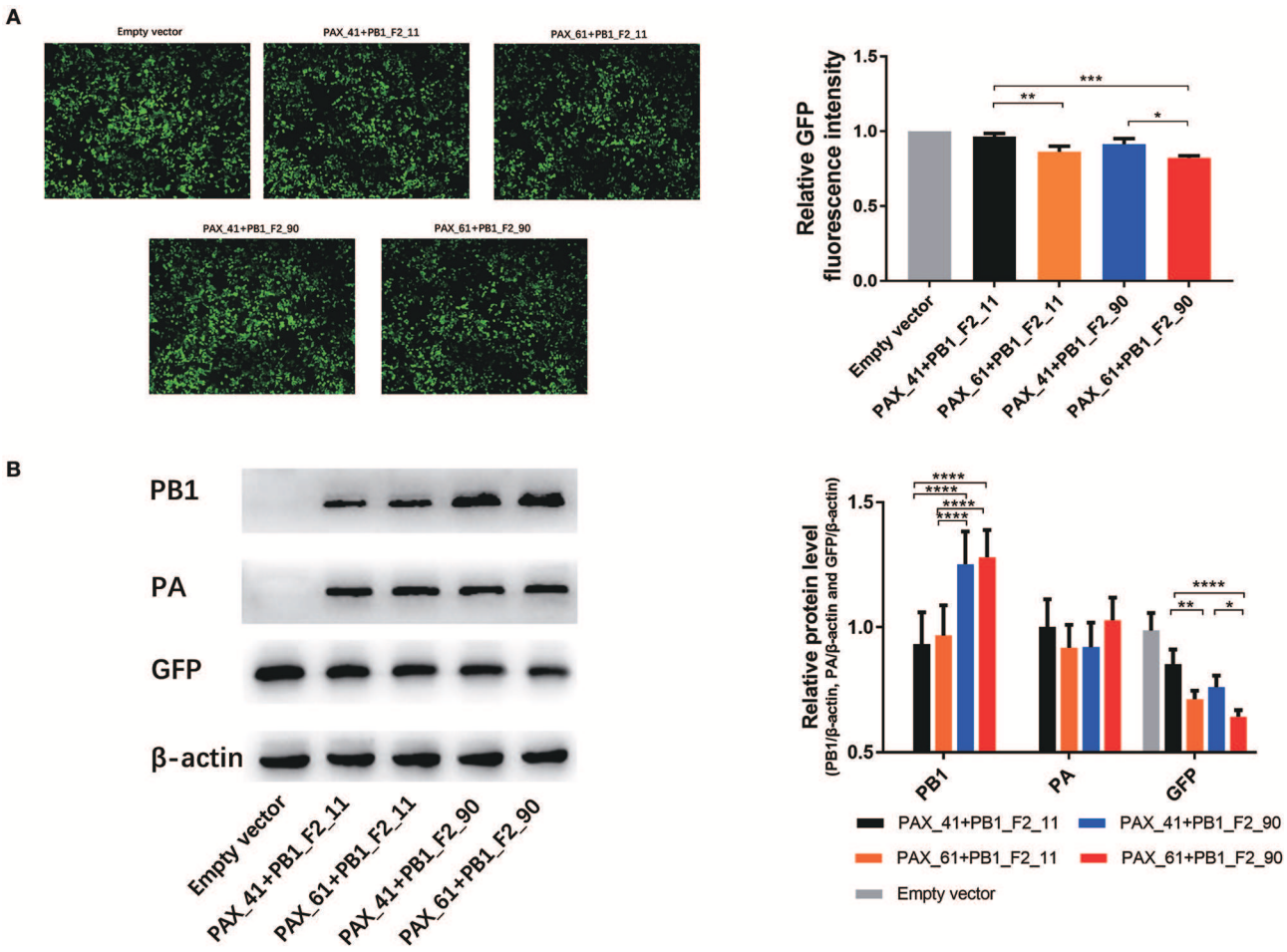

FIGURE 4 | Effects of different expression of PA-X and PB1-F2 proteins on cotransfected GFP expression. 293T cells were cotransfected with the GFP expression plasmid and the indicated PA and PB1 expression plasmids encoding different PA-X and PB1-F2 forms. **(A)** Fluorescence images of GFP expression were captured 24 h post-transfection with the indicated PA and PB1 expression plasmids. The control group was cotransfected with the GFP expression vector along with empty vectors. Relative GFP fluorescence intensity was determined with flow cytometry and data were presented after normalization against the control group (taken as 100%). Three independent experiments were conducted. **(B)**. Expressed PB1, PA, and GFP protein were determined by Western blot analysis. β-actin protein was used as a loading control. The protein bands were quantified by densitometry. Relative protein levels of PB1, PA, and GFP as compared with β-actin are shown as histograms. Differences between each group were considered statistically significant at *p*-values of <0.05. The results shown are representative data from three independent experiments. The significant differences between the groups were marked with asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

significantly lower ($p < 0.05$) than PAX_41+PB1_F2_11. Furthermore, a modest decrease in GFP expression was observed after replacing truncated PB1-F2 with full-length protein (**Figure 4**). These data suggest that the expression of full-length or truncated PB1-F2 may influence the shutoff activity of PA-X in GD1057 viruses.

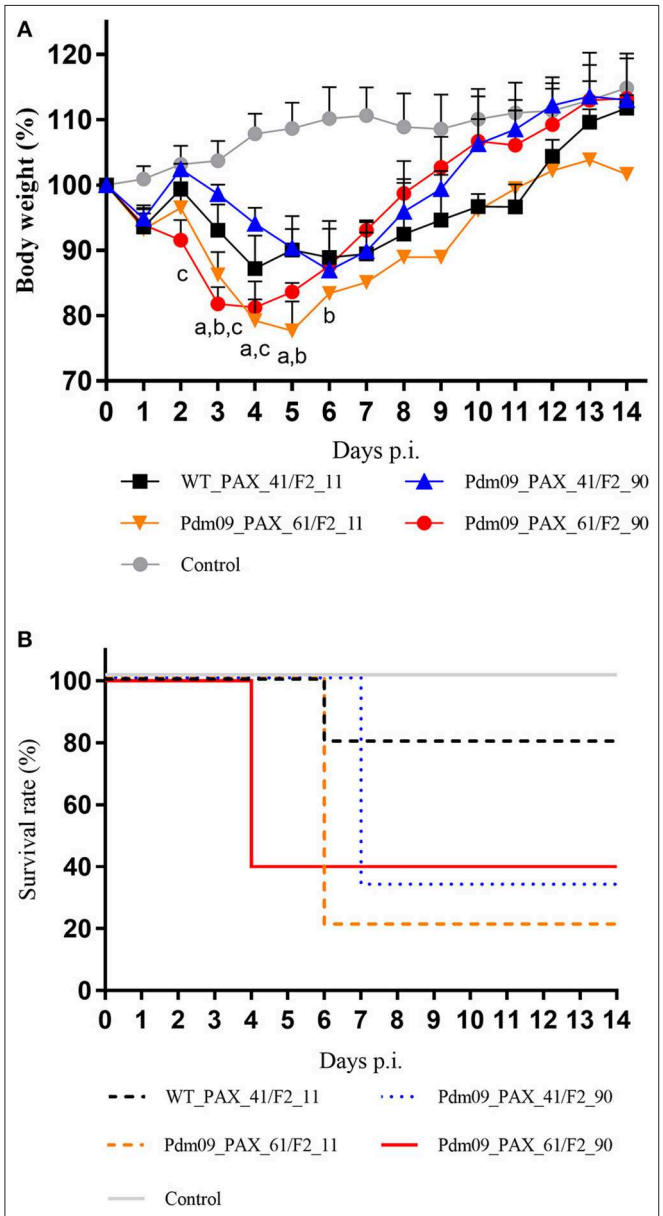

FIGURE 5 | Virulence of different reconstructed viruses in BALB/c mice. **(A)** The body weight of the mice was monitored and recorded until 14 dpi and the results are presented as percentage of the weight on the day of inoculation (day 0). Points of each group show the means and error bars (SD) and significant differences ($p < 0.05$) between infected groups are marked by a, b, and c (a: WT_PAX_41/F2_11 and Pdm09_PAX_61/F2_11; b: WT_PAX_41/F2_11 and Pdm09_PAX_41/F2_90; c: WT_PAX_41/F2_11 and Pdm09_PAX_61/F2_90). **(B)** Survival rates of each experimental group are presented in percentages.

Full-Length PB1-F2 and PA-X Proteins Enhance Viral Pathogenicity During the Early Infection Stage in Mice

To investigate the effects of different forms of PA-X and PB1-F2 proteins on the virulence of the Pdm09 virus *in vivo*, mice from each group were intranasally inoculated with 10^6 $TCID_{50}$ of the corresponding viruses and monitored daily for clinical signs and weight loss. Classic clinical signs of influenza could be observed in all infected mice from the experimental groups, such as depression and reduced activity. Furthermore, weight loss could be measured at 3 dpi when compared with the control animals. It is obvious that viruses (Pdm09_PAX_61/F2_11 and Pdm09_PAX_61/F2_90) expressing full-length PA-X protein induced more body weight loss (79.23 and 81.25% by 4 dpi, respectively) than viruses expressing truncated proteins (WT_PAX_41/F2_11, 87.22% by 4 dpi and Pdm09_PAX_41/F2_90, 86.92% by 6 dpi). It was notable the viruses expressing full-length PB1-F2 and PA-X showed more virulence compared with other three viruses from the 1st to the 4th dpi (**Figure 5A**). To the survival rate, Pdm09_PAX_61/F2_90 exhibited 60% mortality on 4 dpi, which was much higher compared with the other three viruses (**Figure 5B**).

Virus titers in the lungs of the infected mice were measured by $TCID_{50}$, and the results (**Table S9**) demonstrated that Pdm09_PAX_61/F2_90 grew to a significantly higher titer 1 dpi but reached a lower titer 5 dpi when compared with the WT_PAX_41/F2_11 and Pdm09_PAX_61/F2_11 viruses (**Figure 6**).

The pathogenicity induced by the reconstructed viruses was further demonstrated by histopathology analysis of the lung tissues collected at 5 dpi. Typical influenza pneumonia could be observed in all four infection groups. Viruses

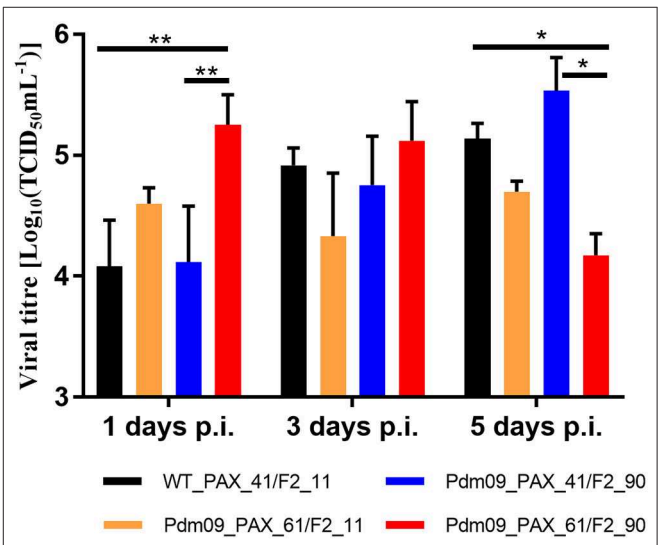

FIGURE 6 | Virus titers of rescue viruses in mouse lungs were determined at 1, 3, and 5 dpi by $TCID_{50}$ in MDCK cells. Values are the mean ± SD of the results from three mice. The significant differences between groups are marked with asterisks (*$p < 0.05$, **$p < 0.01$).

expressing full-length PA-X protein induced more lesions than viruses with a truncated protein and produced more cellular infiltration and inflammatory consolidation, indicated that the full-length PA-X protein increased viral virulence in mice compared with the truncated version (**Figure 7**). Moreover, the Pdm09_PAX_61/F2_90 virus induced the most severe lesions among four viruses; the bronchioles were saturated with neutrophils, and the adjacent alveolar lumina were obviously expanded by lymphocytes and histocytes, indicating a longer duration of lung inflammation. These results suggested that simultaneous expression of full-length PB1-F2 and PA-X proteins enhanced the pathogenicity of the GD1057 virus in the early infection stage in mice.

PB1-F2 and PA-X Proteins Simultaneously Regulate the Inflammatory Response During the Early Infection Stage in Mice

To further estimate the immune response caused by different expression patterns of the PA-X and PB1-F2 proteins, cytokine genes related to the inflammatory response in the lungs of infected mice were determined by qPCR. For the PA-X proteins, the Pdm09_PAX_61/F2_11 virus suppressed the expression levels of NLRP3, IL-18, and IL-1B at 1 dpi and increased the expression levels of caspase-1, IL-1B and TNF-a at 3 dpi when compared with WT_PAX_41/F2_11. For the PB1-F2 proteins, the Pdm09_PAX_41/F2_90 virus increased the levels of NLRP3, caspase-1, IL-1B, and IL-6 at 1 dpi and decreased the levels of IL-18 and TNF-a at later time points (3 dpi). Interestingly, the Pdm09_PAX_61/F2_90 virus upregulated the expression levels of IL-18 and TNF-a at 1 dpi when compared with Pdm09_PAX_61/F2_11. Furthermore, the Pdm09_PAX_61/F2_90 virus expressed higher levels of IL-18 and TNF-a at 1 dpi and higher levels of NLRP3 and TNF-a at 3 dpi when compared with the PAX_41/F2_90 virus (**Figure 8**). These data indicated that single expression of the truncated PA-X protein could suppress the early cytokine response, and single expression of PB1-F2 resulted in an increased early inflammatory response in the lungs of infected mice when compared with the wild type virus. Interestingly, following simultaneous expression of full-length PA-X and PB1-F2 proteins, some of the upregulated gene (NLRP3, IL-1B, and IL-6) expression induced by the full-length PB1-F2 was suppressed by the full-length PA-X. However, the full-length PB1-F2 protein could neutralize the suppression effect caused by the full-length PA-X protein and enhanced the expression level of some cytokine response genes, such as IL-8 and TNF-a. These results indicated that the PA-X and PB1-F2 proteins were simultaneously regulating the host immune response.

DISCUSSION

Since the PA-X protein was discovered in 2012, multiple characteristics of this protein have been described in detail, including its effects on viral pathogenicity and modulation of

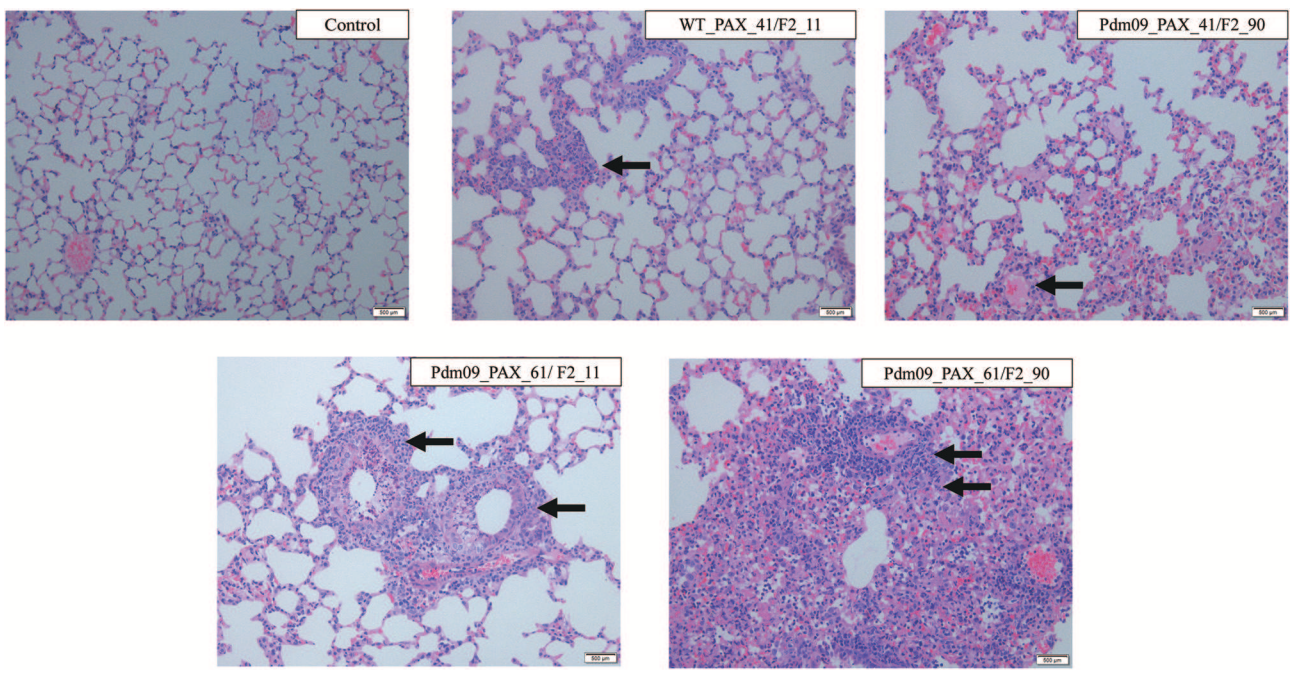

FIGURE 7 | Histopathological changes of mouse lungs infected with the indicated viruses at 5 dpi. Negative control group: No lesions in the bronchiole could be observed. WT_PAX_41/F2_11 and Pdm09_41/F2_90: A small number of neutrophils were present in the bronchiole lumen and there was mild inflammatory consolidation. Pdm09_61/F2_11: The bronchiole was filled with neutrophils and there was moderate mild inflammatory consolidation. Pdm09_61/F2_90: The bronchiole was filled with neutrophils and abundant inflammatory consolidation could be observed. The infiltration of neutrophils was marked by arrows.

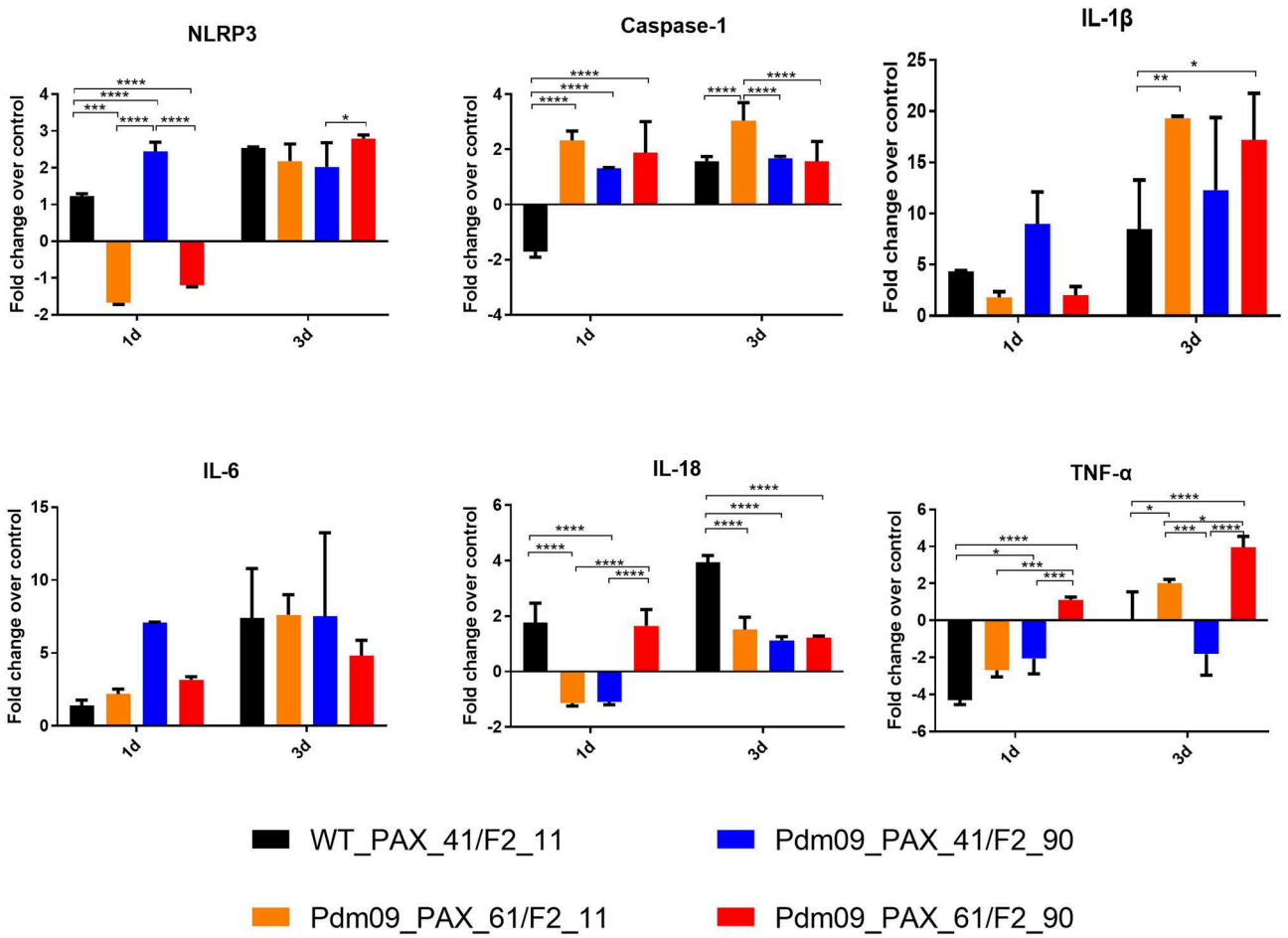

FIGURE 8 | The expression levels of pro-inflammatory cytokines mRNA in the lungs of the mice infected with the reconstructed viruses. The mRNA expression levels of the indicated mouse cytokines were quantified by qRT-PCR. The expression fold change of each group was calculated relative to the control group after normalization to the internal control GAPDH gene using the 2-ΔΔCt method. Data represent the mean ± SD of three mice in each group on the days indicated. The asterisks (*) indicate a statistically significant difference between groups (*$p < 0.05$. **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

the host immune response and the host shutoff activity that suppresses host protein synthesis (Gao et al., 2015b; Hu et al., 2018). Another well-studied virulence factor is the PB1-F2 protein, which has been demonstrated to regulate antiviral innate immunity, enhance viral polymerase activity, induce cell death and alter virus virulence *in vivo* (Kamal et al., 2017). Functions of the PA-X and PB1-F2 proteins in the 2009 pH1N1 virus were individually studied by several researchers (Hai et al., 2010; Pena et al., 2012; Gao et al., 2015a; Lee et al., 2017). However, the combined effects of these two proteins are still unclear. In contrast to previous studies, we investigated the combined effects caused by different forms of the PA-X (232 or 252 aa) and PB1-F2 proteins (11 or 90 aa) in the 2009 pH1N1 virus in regard to viral replication, pathogenicity and host immune response.

A previous study has reported that the full-length PA-X protein from the Cal/09 virus leads to more efficient replication in cells, more viral polymerase activity and greater pathogenicity in mice compared with the truncated version (Lee et al., 2017). For the PB1-F2 protein, it has been indicated that the single restored truncated PB1-F2 protein of Cal/09 only has minimal effects on the replication efficiency and pathogenicity in mice and swine (Pena et al., 2012). Our *in vitro* results of single restored PA-X or PB1-F2 proteins were consistent with previous findings. When we simultaneously expressed both the full-length PA-X and PB1-F2 proteins in the 2009 pH1N1 virus, there was a significant increase of viral growth efficiency in both MDCK and PK-15 cells in the early infection stage when compared with the other three viral groups. Furthermore, a virus with full-length PA-X and PB1-F2 proteins only slightly increased the polymerase activity but significantly improved the viral RNP expression level when compared with the virus expressing full-length PA-X and truncated PB1-F2. These results indicated that the higher early viral replication induced by the presence of full-length PA-X and PB1-F2 proteins was mainly due to increased expression of the viral RNP components.

Our *in vivo* experiments exhibited consistent results with the *in vitro* experiments in that virus expression of the full-length PA-X and PB1-F2 proteins exhibited greater pathogenicity

(weight loss, mortality and pathology) than the other three viruses in the early infection stage of mice. Our results indicated that the expression of full-length PA-X and PB1-F2 proteins enhanced viral replication *in vitro* and pathogenicity *in vivo* during the early infection stage.

Both PA-X and PB1-F2 proteins could regulate the host immune response. The expression of PA-X protein could suppress the inflammatory reactions. Lee et al. (2017) proved that the full-length PA-X suppressed the inflammatory response compared with the truncated one in Cal/09 virus infection. PB1-F2 proteins have been shown to interfere with the immune response through direct interaction with mitochondrial antiviral signaling (MAVS) protein and other components of the MAVS system (Le Goffic et al., 2011; Varga et al., 2012; Yoshizumi et al., 2014), resulting in an early inflammatory response (Pena et al., 2012). In the present study, our results were consistent with previous research in that single restored PA-X could suppress cytokine and chemokine responses in early infection and expression of full-length PB1-F2 by itself could increase the expression level of genes related to cytokines and chemokines.

Interestingly, after comparing data from viruses that contain full-length PA-X and PB1-F2 with data from the other three reconstructed viruses, our results indicated that the early inflammatory response caused by full-length PB1-F2 could be suppressed by full-length PA-X. However, simultaneous expression of PA-X and PB1-F2 enhanced the expression level of IL-18 and TNF-a during the early infection period, although individual expression of full-length PA-X or PB1-F2 could decrease the expression level of these two genes. These results indicated that the PA-X and PB1-F2 proteins could simultaneously regulate the host immune response. Detailed mechanism of the interplay between the PA-X and the PB1-F2 proteins warrants further investigation.

Influenza virus infection leads to the suppression of global host protein synthesis in infected cells, also known as host shutoff activity (Levene and Gaglia, 2018). The influenza host shutoff activity results in a decrease in the host innate immune response and alters ribosome activity toward translation of viral mRNAs. After Jagger et al. (2012) first demonstrated that PA-X can suppress the gene expression of cotransfected plasmids, several studies have showed that PA-X makes a contribution to influenza host shutoff activity in different viral subtypes in multiple animal models (Gao et al., 2015b). Gao et al. (2015b) further demonstrated that the additional 20 aa from the C-terminal end of PA-X protein has a strong shutoff activity. In contrast to the PA and PA-X proteins, there has been no report about PB1 and PB1-F2 proteins being involved in host shutoff effects. Our results showed that the expression of full-length PA-X increased the host shutoff activity as previous research (Gao et al., 2015b). Of note, prolonging the truncated PB1-F2 to full-length form may slightly enhance the host shutoff activity induced by PA-X in the GD1057 virus. Because of PB1-F2 host and strain specific patterns, further research is needed to unveil the mechanism between PA-X and PB1-F2.

In summary, our research indicated that the simultaneous expression of full-length PA-X and PB1-F2 proteins in the 2009 pH1N1 virus increased viral replication during the early infection stage *in vitro*, likely via promoting the expression of the viral RNP complex protein, and enhancing viral virulence *in vivo* mainly through simultaneously mediated host immune response. Moreover, our research indicated that different forms of PB1-F2 may have impacts on the host shutoff activity of PA-X protein in GD1057. Our study provides a better understanding of the functions of different forms of PA-X and PB1-F2 proteins in regard to viral pathogenicity and host immune response.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

JM, ShuL, KL, and XW conducted the experiments. JM analyzed the data and wrote the paper. ShoL designed the experiments and revised the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2019.00315/full#supplementary-material

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BRIEF RESEARCH REPORT
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In vitro Interactions of Chicken Programmed Cell Death 1 (PD-1) and PD-1 Ligand-1 (PD-L1)

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In the present study, we determined the *in vitro* characteristics and binding interactions of chicken PD-1 (chPD-1) and PD-L1 (chPD-L1) and developed a panel of specific monoclonal antibodies against the two proteins. ChPD-1 and chPD-L1 sequence identities and similarities were lower compared with those of humans and other mammalian species. Furthermore, in phylogenetic analysis, chPD-1 and chPD-L1 were grouped separately from the mammalian PD-1 and PD-L1 sequences. As in other species, chPD-1 and chPD-L1 sequences showed signal peptide, extracellular domain, a transmembrane domain and intracellular domain. Based on the three dimensional (3D) structural homology, chPD-1, and chPD-L1 were similar to 3D structures of mammalian PD-1 and PD-L1. Further, Ig V domain of chPD-1 and the Ig V and Ig C domains of chPD-L1 were highly conserved with the mammalian counterparts. *In vitro* binding interaction studies using Superparamagnetic Dynabeads® confirmed that recombinant soluble chPD-1/PD-L1 fusion proteins and surface chPD-1/PD-L1 proteins interacted with each other on COS cells. Two monoclonal antibodies specific against chPD-1 and five antibodies against chPD-L1 were developed and their specific binding characteristics confirmed by immunofluorescence staining and Western blotting.

Keywords: chPD-1, chPD-L1, monoclonal antibodies, cancer, pathways

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INTRODUCTION

Programmed death 1 (PD-1; CD279) is a co inhibitory immunoreceptor molecule belonging to the CD28/CTLA-4/ICOS/B7 immunoglobulin (Ig) superfamily (Ishida et al., 1992; Zhang et al., 2004). PD-1 is expressed on activated T cells, B cells, NKT cells, and myeloid cells (Freeman et al., 2006). PD-1 interacts with its specific ligand, Programmed death ligand 1 (PD-L1; CD274), which delivers inhibitory signal that leads to suspension of immune response by inducing apoptosis, anergy, unresponsiveness, and functional exhaustion of T cells (Freeman et al., 2006; Shi et al., 2011). PD-L1 is broadly expressed on both professional and non-professional antigen presenting cells (APCs), and lymphoid and non-lymphoid tissues (Greenwald et al., 2005; Okazaki and Honjo, 2006).

Immune T cell exhaustion is a state of ineffective T cell response that occurs during chronic (latent) viral infections and cancer (Freeman et al., 2006; Wherry, 2011). PD-1 and PD-1/PD-L1 pathways play a key role in the T cell exhaustion (Freeman et al., 2006; Nakamoto et al., 2009; Wherry, 2011). Recent studies have shown that targeting the PD-1 and PD-L1 pathways and PD-1/PD-L1 checkpoints with monoclonal antibodies are promising to reverse the exhausted T-cell

response in chronic viral infections and in the treatment of various types of cancer (Blackburn et al., 2009; Nakamoto et al., 2009; Brahmer et al., 2012; Topalian et al., 2012).

In veterinary virology or cancer research, there are a few reports on the PD-1 and PD-L1 homologs in cats, pigs and bovines, however their interactions and potential applications for immunotherapy have not been studied in detail (Jeon et al., 2007; Ikebuchi et al., 2011; Maekawa et al., 2014; Zhu et al., 2017). In the avian species, although there is one report on the chicken homologs chPD-1 and chPD-L1 (Matsuyama-Kato et al., 2012), detailed studies on their biological characteristics and interactions have not been carried out. Moreover, no specific monoclonal antibodies (mAbs) are available against chPD-1 and chPD-L1. In the present study, we describe the molecular characterization of chPD-1/chPD-L1 and their specific interactions, as well as report on the successful generation of specific mAbs that will be valuable in examining future roles in chronic infections and cancer in chickens.

MATERIALS AND METHODS

Cell Culture and Transfection

Monkey kidney cell-derived COS cells and chicken fibroblast cell-derived DF-1 cell were used for the *in vitro* characterization of chPD-1 and chPD-L1. COS and DF-1 cells are well-characterized mammalian and chicken cell lines, respectively. COS and DF-1 cells were grown in high glucose Dulbecco's Modified Eagles Medium (DMEM) with Glutamax, supplemented with 10% fetal calf serum, 1,000 U/ml penicillin and 1 mg/ml streptomycin. DF-1 cells are continuous cell lines of EV-0 chicken embryo fibroblasts. COS and DF-1 cells were transfected by using Lipofectamine 2000 reagent (Invitrogen, Karlsruhe, Germany). COS cells were used for immunofluorescence and Western blot assays. DF-1 cells were used for immunofluorescence staining.

Characteristics of chPD-1 and chPD-L1

The mammalian orthologs of PD-1 and PD-L1 sequences were retrieved from the NCBI database and multiple sequence alignments were performed using MEGA 6.06. The amino acid sequences of chPD-1 and chPD-L1 were submitted to the Iterative Threading ASSEmbly Refinement (I-TASSER) database online server (https://zhanglab.ccmb.med.umich.edu/I-TASSER/) to identify the predictive 3D structural models (Yang et al., 2015). Further, the 3D models accuracy was predicted in the ModFOLD 6 (http://www.reading.ac.uk/bioinf/ModFOLD/ModFOLD6_form.html) model quality assessment server. The multiple aligned sequences were submitted to ESPript 3.0 (http://espript.ibcp.fr/ESPript/ESPript/index.php) analysis, using I-TASSER database server (https://zhanglab.ccmb.med.umich.edu/I-TASSER/) generated chPD-1 and chPD-L1 protein structures (PDB) as the templates to predict secondary structures of chPD-1 and chPD-L1 (Robert and Gouet, 2014).

Abbreviations: chPD-1, Chicken Programmed death 1; chPD-L1, Chicken Programmed death ligand 1; RT-qPCR, Real time quantitative PCR.

Construction of Expression Constructs of chPD-1 and chPD-L1

The full-length cDNA of chPD-1 and chPD-L1 were amplified by PCR using primers designed from the predicted sequences of the genes in the NCBI databases (**Supplementary Table 1**). Plasmid pKW06 was used to construct the full length chicken PD1 and PDL1. For total RNA extraction, concanavalin A (Con A) (Sigma-Aldrich, Poole, UK)-stimulated chicken splenocytes were prepared, essentially as described previously (Kaspers et al., 1994). Then, total RNA was extracted using RNeasy mini kit (QIAGEN, Crawley, UK), according to the manufacturer's instructions. First strand synthesis used Superscript III (Invitrogen). After denaturation of the reverse transcriptase at 70 °C for 15 min, 1 μl of the reaction was used in a 50 μl volume polymerase chain reaction (PCR) containing 1 μM dNTP, 10 μM of each primer and 0.625 U of Taq DNA polymerase (Invitrogen). Each cDNA of chPD-1 and chPD-L1 were cloned into pGEM-T vector and sequences confirmed from three independent clones on each strand.

To obtain soluble forms of chPD-1 and chPD-L1, their extracellular domains were identified from comparison of their human and mouse orthologs using the SMART prediction program (http://smart.embl-heidelberg.de/). Primers containing restriction enzyme sites were designed to flank the extracellular domains of chPD-1 and chPD-L1 (**Supplementary Table 1**). The extracellular domains of chPD-1 and chPD-L1, were amplified and cloned initially into the NhoI and NheI sites of pGEM-T vector (Promega, Southampton, UK), and subsequently subcloned into pKW06 (Staines et al., 2013), to generate the chPD-1-human-IgG1Fc and chPD-L1-human-IgG1Fc fusion constructs to produce the soluble COOH-human Fc-tagged recombinant proteins. All the cloning steps were confirmed by sequence analysis. Surface expression recombinant constructs pKW06-chPD-1 and pKW06-chPD-L1 containing the full-length genes of chPD-1 and chPD-L1, respectively, were also generated.

Generation of Monoclonal Antibodies Against chPD-1 and chPD-L1

Monoclonal antibodies were produced against the recombinant chPD-1 and chPD-L1. Immunizations and generation of hybridomas were carried out by the DC biosciences, Dundee, Scotland (https://www.dcbiosciences.com/). Hybridomas that cross react with chPD-1 and chPD-L1 were initially selected based on dot blot ELISA (data not shown).

Immunofluorescence Staining

COS and DF-1 cells (1×10^6) were plated in a six-well culture plates. Recombinant expression constructs pKW06-chPD-1, pKW06-chPD-L1, pKW06-chPD-1-human-IgG1Fc, and pKW06-chPD-L1-human-IgG1Fc were transfected into COS cells (90–95% confluence) by Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany). COS cells were fixed in 4% paraformaldehyde [30 min, Room temperature (RT)] and permeabilized with 0.1% Triton X-100 (15 min, RT). After blocking in 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 30 min, cells were

incubated (1 h, 37°C) with mouse monoclonal anti-chPD-1 and chPD-L1 antibodies (1:500 in 5% BSA). After washing the cells three times to remove any unbound antibodies, cells were incubated (1 h, 37°C) with Alexa 568 (COS cells)/488 (DF-1 cells)-conjugated goat anti-mouse antibodies (1:200 in 5% BSA). Finally, after washing, cells were stained (10 min, RT) with DAPI (1:10000) and viewed by using a Leica (Wetzlar, Germany) TCS SP2 confocal laser-scanning microscope. The experiments were performed in triplicate.

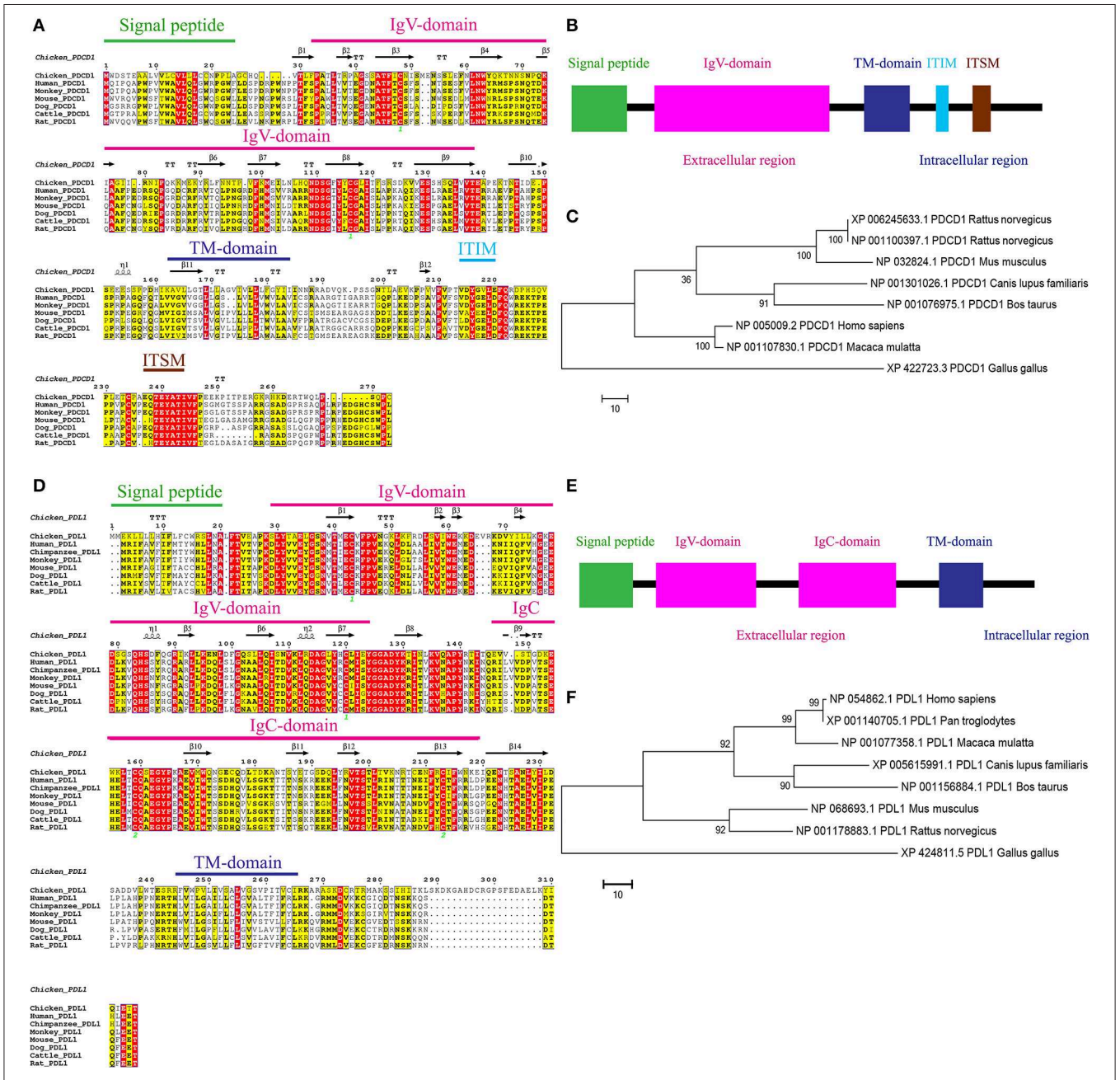

FIGURE 1 | Alignment of the amino acid sequences of chPD-1 and chPD-L1 with their mammalian orthologs. **(A,D)** Qualitative analysis of sequence identity and similarity using the ESPript 3.0 online tool. The predicted secondary structures are marked above the alignment (by helices with squiggles, β strands with arrows, and turns with TT letters) and are based on the PD-1 and PD-L1 structural models. Strictly conserved residues are boxed in white on a red background, more conserved residues are boxed in black on a yellow background, and less conserved residues are boxed in black on a white background. Shaded areas represent conservation of amino acid, the darker the shading, the more conserved the residue across species. The green number in Ig V domain of chPD-1 and Ig V and Ig C domains of chPD-L1 indicate that the two cysteine residues that form an intrachain disulfide bridge, respectively. **(B,E)** Predicted functional motifs in chPD-1 contains extracellular, transmembrane and intracellular domains and chPD-L1 contains extracellular, transmembrane and intracellular domains. **(C,F)** Maximum likelihood phylogenetic trees based on amino acid sequences of chPD-1 and chPD-L1 in relation to other animal species. Bootstrap values of 1,000 replicates was assigned for the analysis.

Western Blot Analysis

The supernatants from COS cells transfected with pKW06-chPD-1-human-IgG1Fc and pKW06-chPD-L1-human-IgG1Fc were treated with TruPAGE LDS sample buffer and boiled for 10 min at 95°C. The samples were then loaded on a 4–12% TruPAGE Precast Gel (Bio-Rad) and transferred onto PVDF membranes. Immunoblots were blocked with 5% skim milk (2.5 g skim milk powder was dissolved in 50 ml of TBST) for 1 h at room temperature. Soluble proteins of chPD-1 and chPD-L1 were detected using monoclonal anti-chPD-1 and chPD-L1 antibodies (1:50 in 5% skim milk), after which blots were washed three times in TBST. Then, blots were incubated (1 h, 37°C) with secondary antibody IRDye 680RD goat anti-mouse IgG (1:200 in 5% skim milk). Finally, after washing, blots were visualized using Odyssey Clx (LI-COR).

Binding of Soluble chPD-1 and chPD-L1 Coated Dynabeads® to COS Cells Expressing Respective Ligands

Dynabeads® Biotin Binder are 2.8 μm superparamagnetic beads that binds biotinylated ligands (proteins or antibodies) were used to examine the interactions between chPD-1 and chPD-L1. Dynabeads® Biotin Binder (45 μl) were mixed with 7.5 μg/ml of biotinylated rabbit anti-human IgG, in 1,500 μl of 0.05% PBS-Tween, and incubated on a rotary mixer (1 h, RT). After 1 h incubation, the tube was held against a magnet to attract the conjugated beads and the fluid was removed using a pipette. The conjugated beads were then washed twice with PBS-Tween by magnet as described above. Soluble chPD-1-human-IgG1Fc and chPD-L1-human-IgG1Fc fusion proteins of an estimated concentration of 5 μg/ml were incubated with the Dynabeads on rotary mixer (1 h, RT) and washed two times with PBS-T and one time with PBS. Dynabead-bound fusion proteins were suspended in 500 μl of culture medium containing FCS, and incubated with COS cells transfected with pKW06-chPD-1 and pKW06-chPD-L1 plasmids (1 h, 37°C). Cells were washed and fixed with 1:1 acetone: methanol and stained with Giemsa (2 min, RT). Before imaging on the EVOS digital microscope (ThermoFisher Scientific, USA). The percentage of binding of surface expressed chPD-1 to soluble chPD-L1-human-IgG1Fc and surface expressed chPD-L1 to soluble chPD-1-human-IgG1Fc was determined in 10 randomly selected fields by scoring COS cells with 5 or more beads as positive under Leica (Wetzlar, Germany) DM IRB light microscope. IF staining and confocal microscopy analysis were also performed by using anti-chPD-1 and chPD-L1 monoclonal antibodies as described above.

RESULTS

Cloning and Analysis of the chPD-1 and chPD-L1

To clone chPD-1 and chPD-L1 gene, total RNA was extracted from Con A-stimulated chicken splenocytes, essentially as described previously (Kaspers et al., 1994), and cDNA synthesized. The ORFs of chPD-1 and chPD-L1 was found to be 273 and 315 amino acids in length, respectively (**Figures 1A,D**). The mammalian orthologs of PD-1 and PD-L1 sequences were retrieved from the NCBI database and multiple sequence alignments were performed. ESPript 3.0 server was used to build identities and similarities among the orthologs. ChPD-1

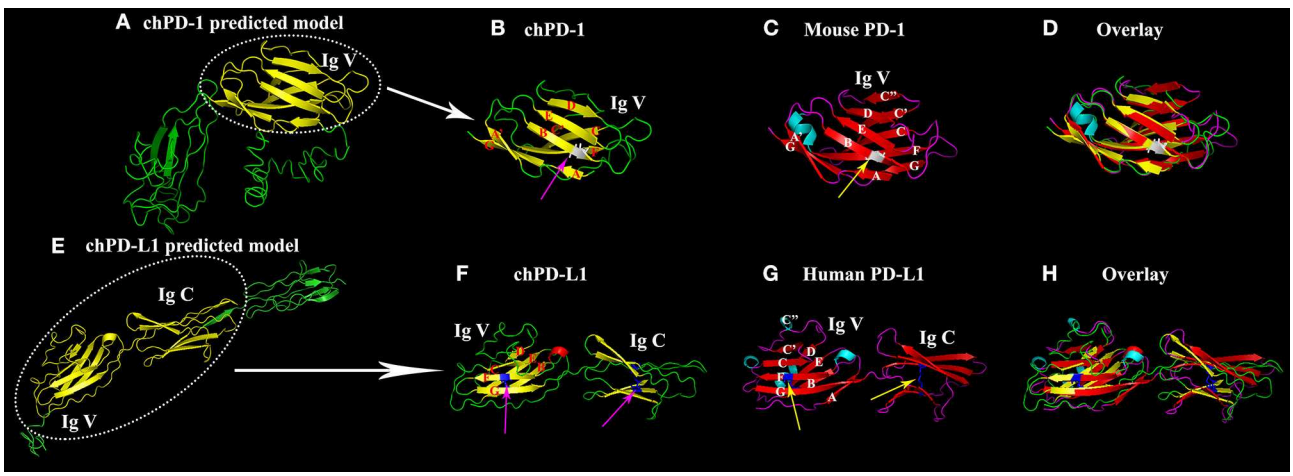

FIGURE 2 | Similarity of the Ig V domain of chPD-1 to the Ig V domain of mouse PD-1 and the Ig V and Ig C domains of chPD-L1 to the Ig V and Ig C domains of human PD-L1. **(A,E)** Predicted homology structural models of chPD-1 and chPD-L1 by I-TASSER software. Although difference in the number of presence of β sheets, the overall structures have identical orientations. **(B–D)** Overlay of Ig V domains of chPD-1 with Ig V mouse PD-1. **(B)** The Ig V domain of chPD-1 is shown in yellow, and **(C)** the Ig V domain mouse PD-1 is shown in red. In mouse PD-1, the two β-sheets are labeled A'GFCC'C" and ABED. In chPD-1, C" and C-terminal of G are absent. The cysteine residues at 48 and 116 form intrachain disulfide bonds are drawn as balls and sticks, and are shown in gray (pink arrow for chPD-1 and yellow arrow for mouse PD-1). **(F–H)** Overlay of Ig V and Ig C domains of chPD-L1 with human PD-L1. **(F)** The Ig V and Ig C domains of chPD-L1 is shown in yellow, and the **(G)** Ig V and Ig C domains of human PD-L1 is shown in red. In human PD-L1, the two β-sheets are labeled AGFCC'C" and BED. In chPD-L1, A, C', and C" are absent. The cysteine residues at 43 and 120 of Ig V domain and at 133 and 225 of Ig C domain form intrachain disulfide bonds are drawn as balls and sticks, and are shown in blue (pink arrow for chPD-L1 and yellow arrow for human PD-L1). Both chPD-1 and chPD-L1 were showed in same view, respectively.

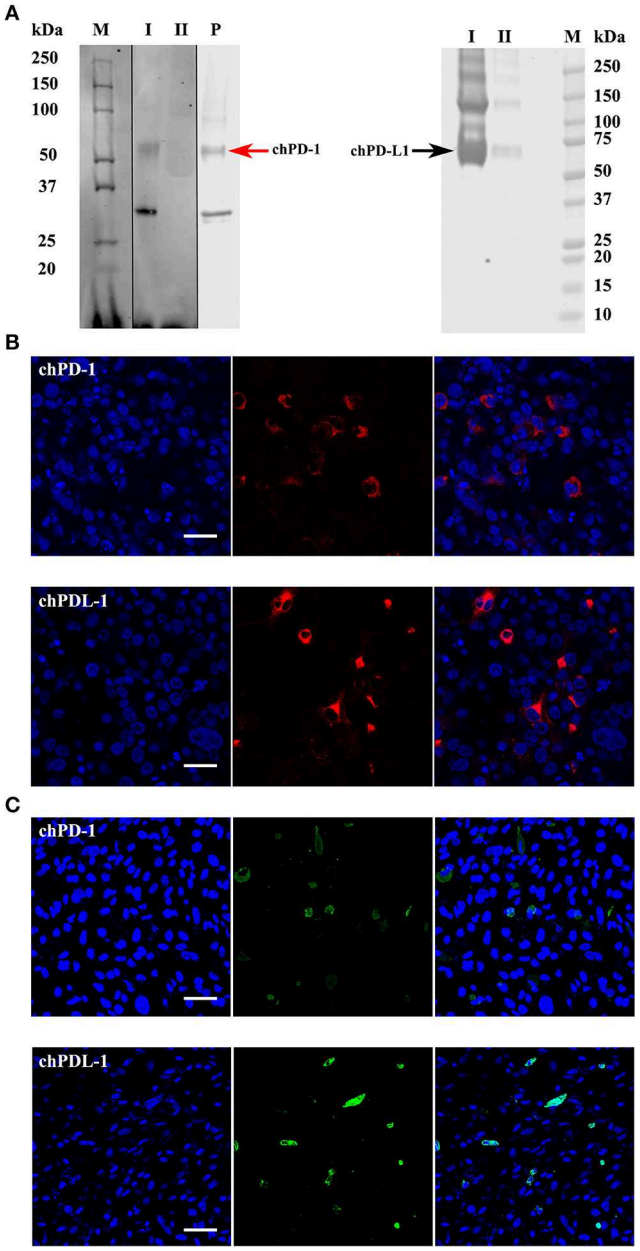

FIGURE 3 | Western blots and confocal micrographs of chPD-1 and chPD-L1. **(A)** The extracellular soluble domains of chPD-1 and chPD-L1 were sub cloned into pkW06 expression vector and expressed as a human Fc-tagged protein, namely, pKW06-chPD-1-human-IgG1Fc and pKW06-chPD-L1-human-IgG1Fc. Proteins were resolved on an SDS 4–12% Bis-Tris gel along with 10% of input protein and western blotted with monoclonal anti-chPD-1 and chPD-L1 antibodies. Images demonstrate that ∼55 kDa (red arrow left direction) for chPD-1 and a band of ∼70 kDa (black arrow right direction) for chPD-L1. I is purified sample, II is supernatant from transfected cells and P is commercial positive control. Dividing black line indicates that intervening lanes have been spliced out. **(B,C)** Representative confocal micrographs illustrating chPD-1 and chPD-L1 in COS (left) and DF-1 (right) cells. Immunofluorescence staining was carried out on COS and DF-1 cells transfected with expression constructs containing pKW06-chPD-1-human-IgG1Fc and pKW06-chPD-L1-human-IgG1Fc plasmids. Cells were fixed after 24–48 h and stained with monoclonal anti-chPD-1 **(A)** and chPD-L1 **(B)** antibodies. Scale bar represents 40 μm.

sequences identities and similarities, respectively, with human (30.76%; 39.56%), cattle (29.3%; 36.66%), mouse (27.83%; 36.99%), dog (27.47%; 36.63%), and rat (27.1%; 35.89%), were relatively lower when compared among mammalian species (**Supplementary Tables 2, 3**). Whereas, chPD-L1 sequences identities and similarities, respectively, with human (38.62%; 50.68%), cattle (36.66%; 47.05%), mouse (38.62%; 48.96%), dog (37.71%; 48.44%), and rat (39.65%; 50.34%) were slightly higher (**Supplementary Tables 2, 3**).

ChPD-1 and chPD-L1 sequences contained the signal peptide, extracellular domain, a transmembrane domain, and intracellular domain, similar to other species (**Figures 1B,E**). PD-1 has composed of an Ig Variable type (V-type) domain in extracellular N-terminal domain between positions 31 to 139 amino acids. The cysteine residues at 48 and 116 that form the intrachain disulfide bond to construct Ig V domain of PD-1, were highly conserved among all the ortholog species. In the Ig V domain of PD-1, ATF (44–46), LNW (61–63), NDSG (109–112), ES (129 and 130), and VTE (137–139) sequences were highly conserved (**Figure 1A**). The intracellular C-terminal PD-1 domain contains a well-conserved two tyrosine (Y) residues, one in an immunoreceptor tyrosine-based inhibitory motif (ITIM: S/I/L/VxYxxL/V) and another in immunoreceptor tyrosine-based switch motif (ITSM: TxYxxL) (**Figure 1A**). The amino acid sequence TEYATIVF around the C-terminal tyrosine is also highly conserved among all the species. PD-L1 is composed of an Ig V-type domain between 29 and 124 amino acids and Ig constant-type (C-type) domain between 148 and 219 amino acids, in extracellular domain (**Figure 1D**). The cysteine residues form the intrachain disulfide bond at positions 43 and 120 to construct Ig V domain and at positions 133 and 225 to construct Ig C domain of PD-L1, were also highly conserved among all the ortholog species. In the IgV domain of PD-1, ATF (44–46), LNW (61–63), NDSG (109–112), ES (129 and 130), and VTE (137–139) sequences were highly conserved (**Figure 1A**). Phylogenetic analysis showed that chPD-1 and chPD-L1 were grouped separately from the mammalian PD-1 and PD-L1 sequences (**Figures 1C,F**).

Homology Models of chPD-1 and chPD-L1

The homology 3D models of chPD-1 and chPD-L1 were built by I-TASSER online tool. For chPD-1, the predicted top model 1 was with: Confidence score (C-score): −3.09, TM-score: 0.37 ± 0.12 and root mean square deviation (RMSD): 15.3 ± 3.4 Å (**Figure 2A**). The best model was used for structural similarity simulation and chPD-1 was found to have highest similarity with the catalytic antibody 28B4, which involved in periodate-dependent oxygenation of sulfide 1 to sulfoxide 2. Furthermore, catalytic antibodies are shown to use for the elucidation of the molecular mechanisms of the immune response and origins of enzymatic catalysis (Yin et al., 2001). For chPD-L1, the predicted top model 1 was with: Confidence score: −1.68, TM-score: 0.51 ± 0.15 and RMSD: 10.1 ± 4.6 Å (**Figure 2E**). The best model was used for structural similarity simulation and chPD-L1 was found to have highest similarity with the SYG-1 and SYG-2 cell adhesion molecules (CAMs). SYG-1 and SYG-2 CAMs have been reported to play diverse role ranging from function in neural development to formation of kidney filtration barrier (Ozkan

et al., 2014). The ModFOLD model quality assessment server 6 was used to assess the quality of the chPD-1 and chPD-L1 3D models, and was CERT confidence with $p < 0.001$ (McGuffin and Roche, 2011; McGuffin et al., 2018).

Cartoon 3D structural diagram of chPD-1 Ig V domain shows a two layer β sandwich, a topology characteristic of Ig V type domains (**Figure 2B**). A superimposition of Ig V domains of chPD-1 and mouse PD-1 showed a good overlap between the structures (**Figures 2B–D**). In a two layer β sandwich of Ig V domain, front A'GFCC'C" and back ABED strands are present in mouse PD-1 (Lin et al., 2008), however C" and C-terminal of G β sheets were absent in chPD-1 (**Figures 2B,C**). Cartoon structural diagram of chPD-L1 IgV and IgC domains reveals a characteristic Ig type topology (**Figure 2F**). IgV and IgC domains of chicken PD-L1 and human PD-L1 superimposition showed a good overlapping structure (**Figures 2F–H**). A two layer β sandwich, front AGFCC'C" and back BED strands are characteristic of Ig V domain of human PD-L1 (Lin et al., 2008), but A, C' and C" were absent in chPD-L1 (**Figures 2F,G**).

Specificity of the mAbs Raised Against chPD-1 and chPD-L1

Initial dot blot ELISA screening showed that the supernatants from two hybridomas reacted with chPD-1 and five hybridomas reacted with chPD-L1 (data not shown). Western blot analysis confirmed that two chPD-1 antibodies and five chPD-L1 antibodies identified in the dot blot ELISA were specific and identified the respective proteins (**Supplementary Table 4**). **Figure 3A** shows a representative Western blot image demonstrating the specific binding of the mAbs to the ∼55 kDa chPD-1 and the ∼70 kDa chPD-L1, respectively. **Figure 3B** is a representative image of immunofluorescence staining of COS cells transfected with pKW06-chPD-1-human-IgG1Fc and pKW06-chPD-L1-human-IgG1Fc plasmids using the specific mAbs (see **Supplementary Files** for individual figures of different hybridomas or their clones). Furthermore, we have confirmed that both the PD-1 and PD-L1 antibodies were specific for chPD-1 and chPD-L1 in chicken DF-1 cells (**Figure 3C**).

chPD-1 and chPD-L1-Specific mAbs Do Not Block the Interaction of the Two Proteins

For the PD-1 and PD-L1 binding assay, the soluble chPD-1-human-IgG1Fc and chPD-L1-human-IgG1Fc fusion proteins were immobilized onto Dynabeads® by using human IgG and incubated on COS cells transfected with the pKW06-chPD-1 and pKW06-chPD-L1 plasmids. After fixation and staining, specific binding was quantified by counting the beads under light microscope. The soluble chPD-1-human-IgG1Fc fusion protein was bound to surface of the pKW06-chPD-L1 transfected COS cells, as observed by rosette formation (**Figure 4A**). Similarly, the soluble chPD-L1-human-IgG1Fc fusion protein was bound to the surface of pKW06-chPD-1 transfected cells with clear rosette formation (**Figure 4A**). It was estimated that $34.89 \pm 6.8\%$ of chPD-L1-human-IgG1Fc fusion proteins and $33.79 \pm 10.3\%$ of chPD-1-human-IgG1Fc fusion proteins showed clear rosette interaction on surface pKW06-chPD-1 and pKW06-chPD-L1 transfected COS cells (**Figure 4B**). The presence of clear rosette between chPD-1 and chPD-L1 was confirmed also by IF staining (**Figure 4C**). Using this binding assay, we examined whether any of the PD-1 or PD-L1-specific mAbs developed in this study interfered with the specific interactions of PD-1 and PD-L1. Despite the ability of the mAbs to bind to the two proteins, they were unable to inhibit or reduce the interaction of chPD-1 and chPD-L1 binding interactions.

DISCUSSION

The immune system plays an important role in the control of viral pathogenesis and tumorigenesis (Cully, 2017; Hashimoto et al., 2018). During chronic viral infections the increased level or duration of stimulation of virus specific CD8 T cells leads to non-functional state called T cell exhaustion (Freeman et al., 2006). Recent studies have shown that PD1 is highly expressed on exhausted T cells, and PD-1/PD-L1 checkpoints are targets of immunotherapy. Currently, in humans, usage of monoclonal antibodies as blockades of PD-1 and PD-L1 pathways, and inhibitors of the PD-1/PD-L1 interaction are gaining momentum (Brahmer et al., 2012; Topalian et al., 2012). However, chPD-1 and chPD-L1 have not been characterized and no specific mAbs have been developed. We have now characterized the chicken PD-1 and PD-L1, studied their binding interactions and developed specific mAbs against chPD-1 and chPD-L1.

The PD-1 and PD-L1 sequence homologies were lower in chPD-1 and chPD-L1 sequences compared with those of the mammalian species (Jeon et al., 2007; Gjetting et al., 2019). The conservation of the ITIM and ITSM motifs in the C-terminal, and highly conserved TEYATIVF indicated that the immune signal regulation associated with PD-1 is highly conserved among species (Okazaki et al., 2001). The stoichiometry between human PD-1 and PD-L1 reported to form a 1:1 complex, chickens may also form similar ratio because the predicted 3D structures of the domains suggested identical configurations (Lin et al., 2008). C-score estimates the quality of predicted models, and −3.09 of chPD-1 and −1.68 of chPD-L1 signifies that both were within the usual confidence range of −5 to 2 (Yang et al., 2015). TM-score and RMSD give information on structural similarity with other structures, and correlation with C-score.

In the present study, we showed that recombinant chPD-1 and chPD-L1 interacted with each other. The strong binding interactions between chPD-1 and chPD-L1 demonstrated that these molecules participate in the suppression of antigen specific immune responses, which is similar to other species (Okazaki et al., 2001; Carter et al., 2002; Maekawa et al., 2014). Furthermore, the development of specific mAbs against the chicken homologs gives the potential for inhibiting the PD-1/PD-L1 interaction pathways for immunotherapy in chronic diseases in avian species. However, non-inhibition of chPD-1 and chPD-L1 interactions by the panel of mAbs developed in this study suggested that the specific antigenic epitopes identified by these mAbs are most likely outside the interacting domains of the PD-1 and PD-L1.

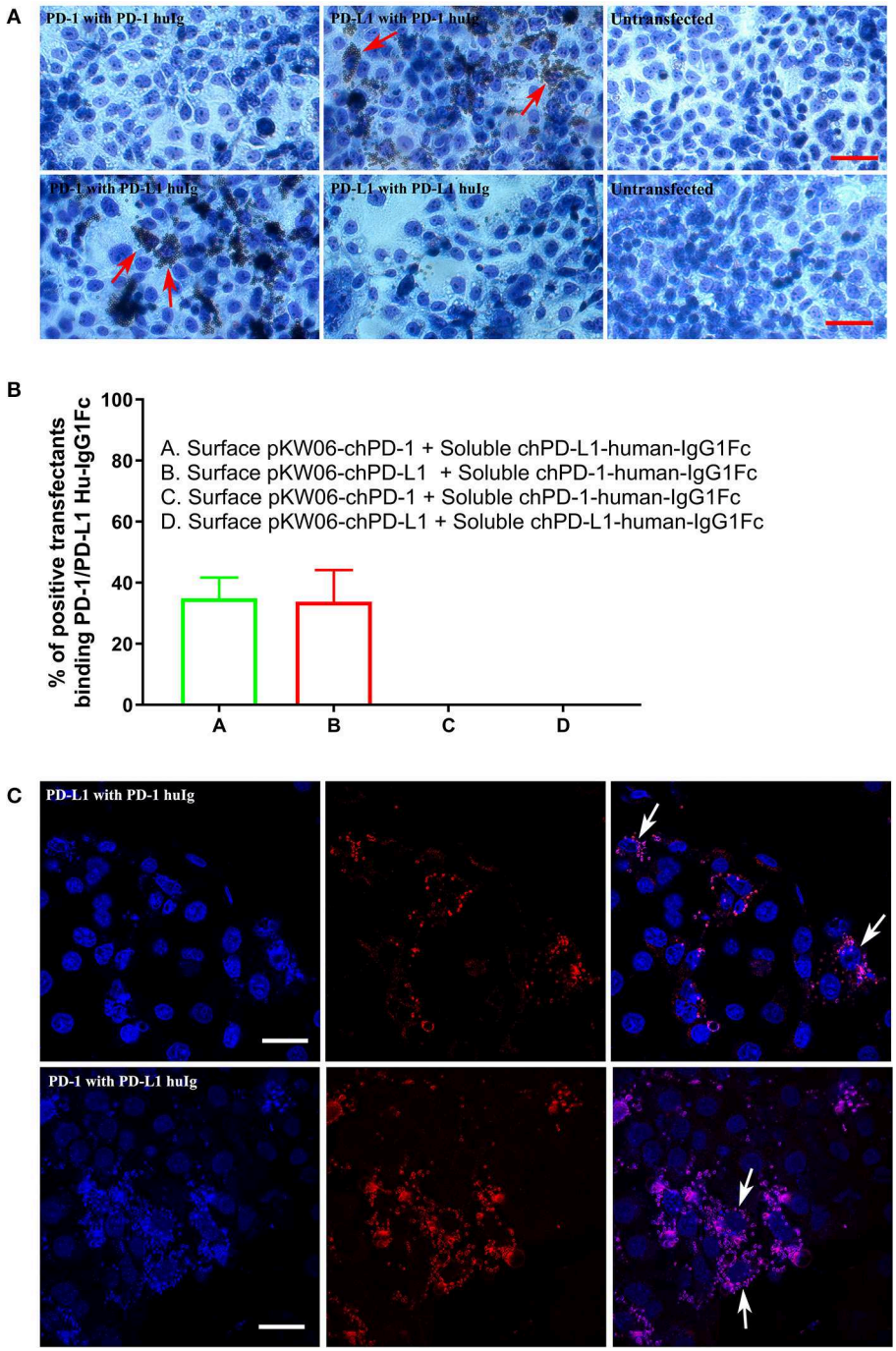

FIGURE 4 | Binding interactions of recombinant soluble chPD-1/PD-L1 with surface chPD-1/PD-L1 on COS cells. **(A)** Representative EVOS images illustrating chPD-1 and chPD-L1 interactions. Images were taken using an EVOS digital microscope. Giemsa staining was carried out on COS cells transfected with surface expression constructs pKW06-chPD-1and pKW06-chPD-L1. Rosette formation was observed when surface pKW06-chPD-L1 interacts with soluble chPD-1-human-IgG1Fc fusion protein (red arrows) and surface pKW06-chPD-1 with soluble chPD-L1-human-IgG1Fc fusion protein (red arrows). No rosettes was observed in surface pKW06-chPD-1 with soluble chPD-1-human-IgG1Fc fusion protein interaction, surface pKW06-chPD-L1 with soluble chPD-L1-human-IgG1Fc fusion protein interaction, and untransfected cells. Scale bar represents 50 μm. **(B)** Percentage of soluble chPD-1/PD-L1-human-IgG1Fc fusion proteins interactions with surface pKW06-chPD-1/PD-L1 were calculated by counting 10 randomly selected fields by scoring COS cells with 5 or more beads as positive. On surface pKW06-chPD-1 and pKW06-chPD-L1 transfected COS cells, 34.89 ± 6.8% of chPD-L1-human-IgG1Fc and 33.79 ± 10.3% of chPD-1-human-IgG1Fc showed clear rosette structures, respectively. Data are represented as means of three independent biological experiments ± standard deviation (error bars). **(C)** Representative confocal photo micrographs illustrating clear rosette (white arrows) formation during chPD-1 and chPD-L1 interactions. Red fluorescence visualizes Dynabeads®. Scale bar represents 40 μm.

Further detailed structural and mutagenesis studies are needed to identify the epitopes of these specific mAbs to understand why they are unable to interfere with the PD-1/PD-L1 interactions.

In conclusion, we describe the characterization of the chPD-1 and chPD-L1 molecules and their interactions. We have also developed a panel of mAbs that specifically identified the chicken PD-1 and PD-L1 homologs. Despite their specific binding to the chPD-1 and chPD-L1, none of the mAbs were able to prevent the interactions of the two proteins. Nevertheless, the results of this work will form the technical basis for future research to explore the role of PD-1/PD-L1 pathway in the latency mechanisms and immunosuppression of Marek's disease and other chronic viral infections of chickens.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

VR, WM, and VN conceived and designed the study, analyzed the data, and drafted the manuscript. VR and WM performed the experiments. YS participated in design and analysis of the results. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Pattern Recognition Receptor Signaling and Innate Responses to Influenza A Viruses in the Mallard Duck, Compared to Humans and Chickens

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Mallard ducks are a natural host and reservoir of avian Influenza A viruses. While most influenza strains can replicate in mallards, the virus typically does not cause substantial disease in this host. Mallards are often resistant to disease caused by highly pathogenic avian influenza viruses, while the same strains can cause severe infection in humans, chickens, and even other species of ducks, resulting in systemic spread of the virus and even death. The differences in influenza detection and antiviral effectors responsible for limiting damage in the mallards are largely unknown. Domestic mallards have an early and robust innate response to infection that seems to limit replication and clear highly pathogenic strains. The regulation and timing of the response to influenza also seems to circumvent damage done by a prolonged or dysregulated immune response. Rapid initiation of innate immune responses depends on viral recognition by pattern recognition receptors (PRRs) expressed in tissues where the virus replicates. RIG-like receptors (RLRs), Toll-like receptors (TLRs), and Nod-like receptors (NLRs) are all important influenza sensors in mammals during infection. Ducks utilize many of the same PRRs to detect influenza, namely RIG-I, TLR7, and TLR3 and their downstream adaptors. Ducks also express many of the same signal transduction proteins including TBK1, TRIF, and TRAF3. Some antiviral effectors expressed downstream of these signaling pathways inhibit influenza replication in ducks. In this review, we summarize the recent advances in our understanding of influenza recognition and response through duck PRRs and their adaptors. We compare basal tissue expression and regulation of these signaling components in birds, to better understand what contributes to influenza resistance in the duck.

Keywords: influenza, duck, reservoir host, innate immunity, tropism

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INTRODUCTION

Influenza A virus (IAV) is a negative sense single stranded RNA (-ssRNA) virus which causes significant disease in both humans and animals. Due to rapid accumulation of mutations during replication, this virus can change surface proteins quickly, thus escape both natural and vaccine-based immunity. These mutations also affect the pathogenicity of individual viral strains.

In chickens especially, IAV can cause severe disease and mortality. The virus is classified as low pathogenic or highly pathogenic avian influenza (LPAI and HPAI, respectively) depending on the severity of disease that it causes in chickens (Alexander et al., 1986; Burggraaf et al., 2014). LPAI strains cause mild symptoms and the birds generally recover within a few days whereas HPAI strains tend to spread systemically and often kill chickens within the first few days of infection.

IAV preferentially replicates in different tissues and organs in the host, and initial infection often depends on the linkage type of terminal sialic acid on glycoproteins expressed on the surface of cells. Viral hemagglutinin (HA) surface proteins bind to glycoprotein-linked sialic acid (SA) on the surface of host cells. The specific linkage of these sialic acids allows the virus to not only become specific to different host species, but also different tissues in these hosts. Humans express sialic acid α-2,6 linked galactose (SA α-2,6-Gal) surface molecules on epithelial cells in the upper airways, which is the site of replication for IAV in humans (Baum and Paulson, 1990; Couceiro et al., 1993). As such, strains of IAV that infect humans replicate in the upper airways. Birds, however, predominantly express SA α-2,3-Gal in their digestive tracts and lungs (Costa et al., 2012). Strains of IAV which are adapted to replicate in birds preferentially bind these receptors over human SA α-2,6-Gal receptors. Chickens also express α-2,6-Gal in their intestinal tracts and lungs, whereas ducks only express these receptors in their lungs. Chickens also have a predominance of SA α-2,6-Gal in their trachea whereas ducks have SA α-2,3-Gal receptor dominance (Kuchipudi et al., 2009). As IAV has been known to jump host species, as is the case of avian IAV jumping to humans, this suggests that chickens may be responsible for propagating avian strains of influenza that can then infect humans. IAV can use other receptors such as phosphoglycans on host cells to gain entry and seems to depend on more than just SA linkages to enter cells (Byrd-Leotis et al., 2019).

Ducks and migratory waterfowl are thought to be the reservoir hosts of IAV, as they appear to have shared a long evolutionary history with the virus (Webster et al., 1992; Taubenberger and Kash, 2010). Indeed, phylogenetic analysis has suggested that avian IAV and circulating mammalian strains of IAV share a recent common ancestor of avian origin. So called "dabbling ducks," or more specifically ducks of the genus *Anas*, are the most frequent host of circulating strains of IAV (Kida et al., 1980; Olsen et al., 2006; Runstadler et al., 2007; Jourdain et al., 2010). For simplicity, we will generalize the term "ducks" to mean mallard ducks (*Anas platyrhynchos*), which also includes the many breeds of domesticated mallard ducks (Zhang et al., 2018). When infected with IAV, ducks generally have no or very mild symptoms, yet surprisingly still replicate and excrete viruses at high titres (Kida et al., 1980). LPAI can replicate in the intestines of ducks for up to 5 days without causing lesions (Daoust et al., 2013). Often called the "Trojan Horse" of infection, these migratory birds can then spread the virus to other ducks in waterways, or to other bird species as they migrate (Kim et al., 2009). HPAI however, preferentially replicates in the lungs of infected ducks, and is more likely to spread systemically in ducks and chickens (Bingham et al., 2009; Vidana et al., 2018). After such a long evolutionary history, the reservoir host likely has evolved adaptations to circumvent damaging effects of prolonged viral replication.

While ducks can control most strains of IAV, some HPAI strains cause significant disease and mortality in ducks, especially those belonging to the H5 subgroup and clade 2.3.2.1 (Sturm-Ramirez et al., 2004; Bingham et al., 2009; Hagag et al., 2015; Haider et al., 2017). It is difficult to generalize, however, because in challenge experiments using viruses belonging to this clade, ducks demonstrated differences in mortality ranging from 100% lethal to no mortality (Kang et al., 2013; Ducatez et al., 2017). Most strikingly, two viruses from the 2.3.2.1 clade that differed by only 30 amino acids showed complete differences in mortality in mallards, with one virus being 100% lethal while the other causing no mortality (Hu et al., 2013). All of these strains are lethal to chickens and many other species. However, some species may show resistance to some strains. Pigeons are resistant to some strains of H5N1, including to strains belonging to clade 2.3.2 (Smietanka et al., 2011; Yamamoto et al., 2012). However, as summarized in a recent review (Abolnik, 2014), pigeons often do not replicate the virus to significant titres and only shed the virus for a short period of time. We also cannot generalize about all ducks as other types of ducks exhibit varied reactions when infected with H5N1 strains of virus. Gadwall, wigeon, and mallard ducks were asymptomatic, while mandarin duck, tufted ducks, ruddy shelducks, and several species of geese and swans showed signs of morbidity and mortality (Gaidet et al., 2010). In another study, swans and ruddy shelducks showed 100% mortality when infected with HPAI H5N1, whereas mallard ducks had an asymptomatic infection (Kwon et al., 2010). Thus, infection and mortality rates differ between different types of ducks. These studies highlight the difficulty in making generalizations about avian influenza studies but can also pinpoint residues contributing to virulence in each host species. What makes mallard ducks so successful at both limiting viral replication of HPAI virus and resisting damage from replicating virus is currently unknown.

When birds are infected with IAV, the first few days seem to be the most important when determining survival vs. succumbing to infection, highlighting the importance of innate immunity as a protective mechanism. We recently reviewed the immune responses of ducks and chickens to IAV (Evseev and Magor, 2019). Birds diverged from mammals about 300 million years ago yet have retained many of the same innate immune mechanisms that mammals use to combat viral infections. When viral or pathogen associated molecular patterns (PAMPS) are detected by the host, they are detected by specific pattern recognition receptors (PRRs) in order to elicit antiviral responses including cytokines, chemokines, and upregulation of antiviral effectors. Both immune and non-immune cells contain these PRRs. PRRs of avian species were previously reviewed in 2013 (Chen et al., 2013), however significant advances have been made since that time. In this review, we summarize recent advances in understanding innate signaling pathways in ducks by looking at the similarities and differences between PRR tissue expression in ducks, chickens, and humans. We also further review new research in characterizing protein function in the

signal transduction platform in order to understand how innate signaling pathways differ or are the same in these three species.

The three important PRR signaling pathways responding to influenza infection include toll-like receptors (TLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (**Figure 1**). TLRs, RLRs, and NLRs can all be found on the cell surface or in cytosolic compartments in the cell. These PRRs all act to recognize influenza viral components such as double stranded RNA (dsRNA), single stranded RNA (ssRNA), and RNA with a 5′ triphosphate overhang (5′pppRNA) (Yoneyama et al., 2004; Okamoto et al., 2017). Many of these PRRs have signaling pathways that converge downstream to produce interferons (IFNs) or proinflammatory cytokines and utilize similar scaffolding and adaptor proteins to amplify this signal. In this review, we compile recent studies on characterization of these influenza sensors, signaling pathways and their downstream effectors in both chickens and ducks.

Basal expression of these PRRs may also allow different tissues to detect IAV infection earlier. To visualize PRR readiness we show basal expression patterns in different tissues in ducks and chickens (**Figure 2**). Tissues studied include immune relevant organs such as the lung, spleen, bursa, thymus, and intestine as well as other organs such as brain, kidney, and heart.

RLR RECEPTORS AND THEIR ADAPTORS

The RIG-I like receptor (RLR) family are select cytosolic RNA helicases which contain conserved DExD/H box domains used in nucleic acid binding (Loo and Gale, 2011). These PRRs sense non-self RNA from viral pathogens. In contrast to other PRRs like TLRS, RLRs are expressed in immune cells as well as in somatic cell types such as epithelium, thus can protect cell types most targeted by viral infection (Uhlen et al., 2015; Francisco et al., 2019). RLRs involved in IAV recognition include retinoic acid inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2) (**Figure 1**). RIG-I and MDA5 share much structural similarity, with both proteins having two caspase activation and recruitment (CARD) domains, a central DEAD helicase domain and a C-terminal repressor domain (RD) (Yoneyama et al., 2005; Zou et al., 2009). While the DExD/H box helicase domain has the ability to use ATP hydrolysis to aid in binding and unwinding viral RNA, the RD has been implicated in self repression (as in RIG-I). CARD domains are involved in relaying the signal to the downstream adaptor, the mitochondrial antiviral signaling protein (MAVS) (Jacobs and Coyne, 2013; Wu and Hur, 2015). LPG2 is lacking the CARD domains that RIG-I and MDA5 possess but shares structural similarity in the

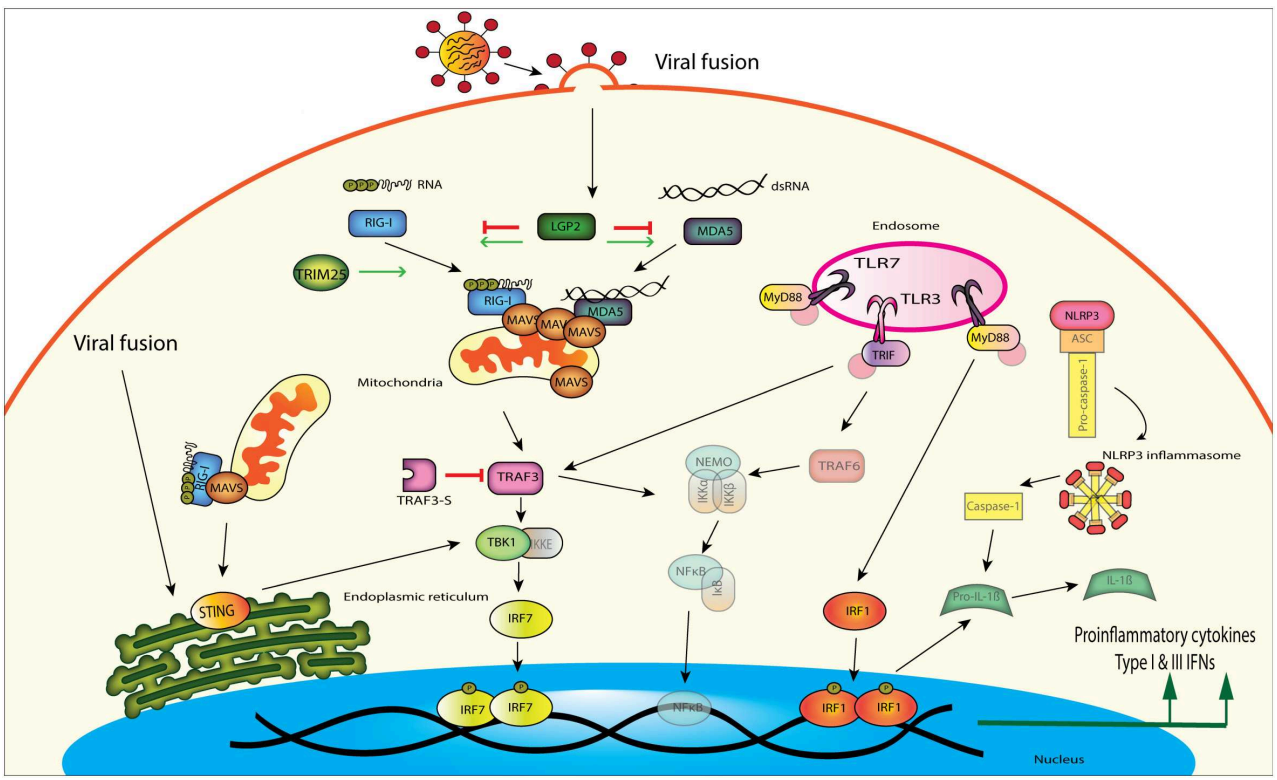

FIGURE 1 | Influenza A virus is detected by several different innate immune signaling proteins in the cell. RIG-I and MDA5 both can bind to viral RNA and signal downstream through MAVS, TBK1, and IRF7. Viral fusion can also be detected by the endoplasmic reticulum localized STING, which then signals through TBK1 to induce interferon production. TLRs located in the endosome can recognize viral RNA and signal through different adaptor proteins to induce proinflammatory cytokines and IFNs. TLR3 uses TRIF as an adaptor protein to amplify signaling through TRAF3 or TRAF6, while TLR7 uses the adaptor MyD88 to signal through TRAF3. TLR signaling through MyD88 can also activate the NLRP3 inflammasome, which increases pro-inflammatory cytokine production through IRF1.

DExD/H box and RD domains (Pippig et al., 2009). The cytosolic sensor MDA5 preferentially recognizes long dsRNA, whereas RIG-I recognizes shorter dsRNA sequences that are produced during IAV replication (Kato et al., 2008). Once these cytosolic sensors recognize viral RNA, a signal is transduced through MAVS to downstream components to induce type I IFN or proinflammatory cytokine production.

RIG-I

RIG-I is the primary sensor of influenza virus in all cells except plasmacytoid dendritic cells. RIG-I detects dsRNA and viral transcriptional intermediates bearing 5′-pppRNA in infected cells (Hornung et al., 2006; Pichlmair et al., 2006; Schmidt et al., 2009). A panhandle structure, formed by binding of complementary regions in the influenza RNA transcript, is detected by RIG-I (Liu G. et al., 2015). Recently transcriptional intermediates called mini viral RNAs of about 80 nucleotides in length have been shown to act as RIG-I ligands (te Velthuis et al., 2018). Notably, it was recently shown that RIG-I detects viral replication not only in the cytoplasm, but also in a nuclear compartment (Liu G. et al., 2018). This may be particularly relevant for influenza detection, since influenza replicates in the nucleus. A recent review considers how dsRNA and viral transcriptional intermediates bearing 5′-pppRNA made in the nucleus are detected by RIG-I in the cytoplasm of infected cells (Liu and Zhou, 2019). It is not known whether RIG-I is capable of nuclear detection in lower vertebrates. In addition, RIG-I (but not MDA5) can act as an antiviral effector protein by directly binding to incoming IAV viral RNA (Weber et al., 2015). RIG-I also has far reaching effects on immune responses. Mice deficient in RIG-I signaling show defects in dendritic cell activation and mobilization, viral antigen presentation and impairment of polyfunctional T cell responses (Kandasamy et al., 2016). More recently, the importance of RIG-I in IAV infection has been questioned. Surprisingly, when RIG-I was knocked out of mice, this did not make mice more susceptible to lethal influenza infection (Wu et al., 2018). These results may stem from mice not being a natural host of IAV or perhaps they rely on different recognition strategies to detect virus.

RIG-I is ubiquitously expressed in human tissues, but ducks have tissue specific basal expression of RIG-I and chickens appear to be missing RIG-I entirely. RIG-I is expressed in most human tissues and does not exhibit tissue specific expression, although there is slightly higher mRNA expression in the thymus, granulocytes, and adipose tissues (Uhlen et al., 2015). A comparison of tissue expression of RLR pathway components between chickens and ducks illustrates the readiness of these tissues to respond to pathogens (**Figure 2A**). In Muscovy ducks, RIG-I is most highly expressed in the trachea and digestive tissues (Cheng et al., 2015a). Chickens appear to have lost RIG-I (Barber et al., 2010). RIG-I gene loss has also been documented in mammals, such as the Chinese tree shrew (Xu et al., 2016). RIG-I knockouts generated in C57BL/6 mice are lethal in the developing embryos (Kato et al., 2005), however this lethality was

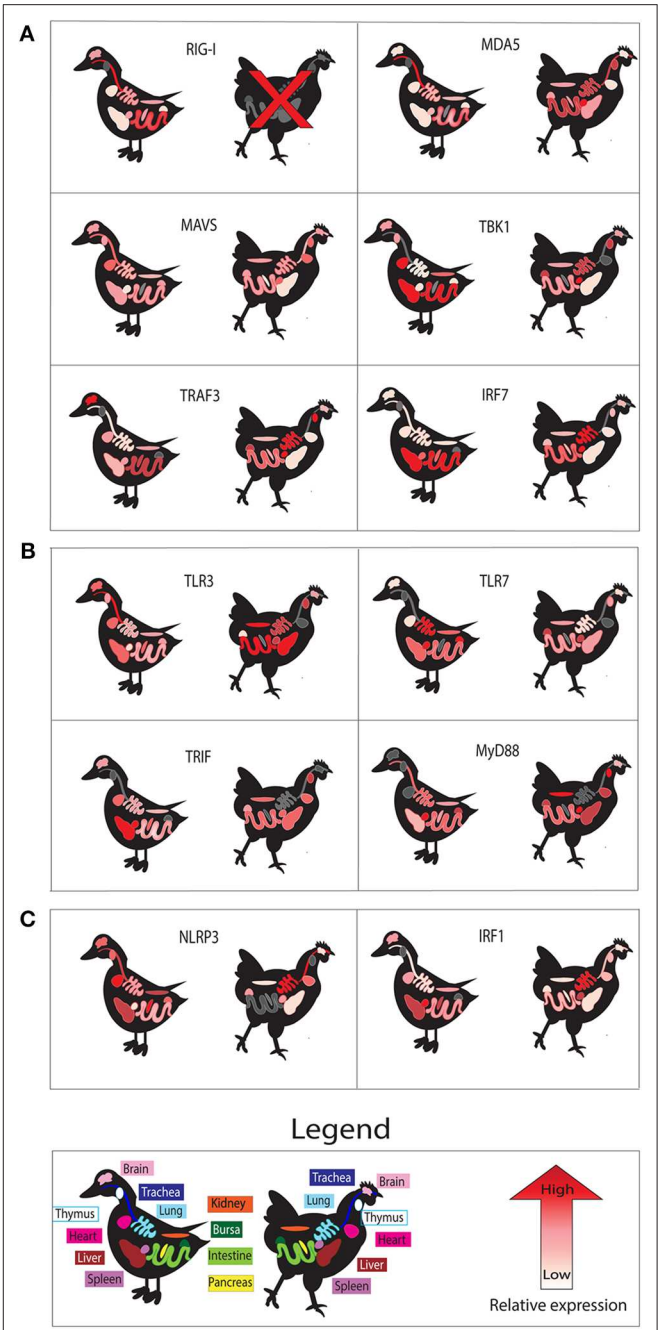

FIGURE 2 | Basal tissue expression of genes in uninfected ducks compared to those in chickens. Tissue expression is shown for components of RLR **(A)**, TLR **(B)**, and NLRP3 inflammasome **(C)** signaling pathways. We show relative expression of each gene studied in those tissues. High relative basal gene expression is denoted by red, while lower expression is indicated by pinks and whites. Gray coloring indicates no data available for the gene in the indicated tissue. All data was extracted from individual studies in this review, and color scales are relative for data from individual studies. All data is for mallard duck, except RIG-I and MDA5, which are Muscovy duck. Data obtained for chicken MDA5, MAVS, and IRF1 were obtained from the chicken atlas (http://biogps.org/), and averages for each tissue in adult chickens were used to estimate relative expression.

not seen in mice with a more complex genetic background (Wu et al., 2018).

Duck RIG-I can function in chicken cells, indicating that chickens have the corresponding downstream signaling components. When we overexpressed duck RIG-I in chicken fibroblasts, the cells could detect RIG-I ligand and produce interferon (Barber et al., 2010). We also showed that chicken cells transfected with duck RIG-I produce more IFN-β, augment expression of numerous ISGs, and restrict influenza virus (Barber et al., 2013). Others have demonstrated that chickens detect IAV through the related RLR, MDA5 (Karpala et al., 2011; Liniger et al., 2012). We have speculated that one reason ducks so successfully control influenza virus while chickens do not is partially because of RIG-I. This has been controversial, and we acknowledge that because RIG-I has not been detected does not prove it does not exist. No disrupted gene has been found to confirm its absence. If chicken RIG-I has significantly diverged from duck RIG-I, it would not be detected through hybridization, or PCR. Likewise, it has also been notably absent from the now extensive transcriptome databases available for chickens and other galliform birds. However, if a chicken RIG-I ortholog is expressed in very low amounts or has a very high GC content, it may be difficult to sequence using standard next generation sequencing technology. An interesting experiment to determine the significance of RIG-I in birds would be to knock RIG-I out of ducks or introduce duck RIG-I into chickens. However, some strains of influenza viruses can kill ducks even in the presence of RIG-I, demonstrating that many other factors contribute to successful defense.

RIG-I is upregulated quickly during influenza infection, with a peak at 24 h and expression returning to normal levels in lung, intestine, and spleen when Pekin ducks are infected with both HPAI and LPAI IAV strains (Fleming-Canepa et al., 2019). In these studies, RIG-I is upregulated much more during HPAI infection than LPAI infection. In Muscovy ducks RIG-I mRNA expression peaked at 2 DPI in brain and spleen, while expression was highest 1 DPI in the lung and bursa (Cheng et al., 2015a). Muscovy ducks are more susceptible to influenza infection than mallard ducks (Phuong do et al., 2011), and this slight delay in RIG-I upregulation may contribute.

MDA5

MDA5 was often thought to be of less importance in IAV infection because of its preference for longer dsRNA, however siRNA knockdown of this host mRNA during IAV infection in mice demonstrated that MDA5 is also an important factor in viral restriction (Benitez et al., 2015). While it appears that chickens have lost RIG-I (Barber et al., 2010), they use the related cytosolic receptor MDA5 to detect IAV and signal through MAVS to induce IFN and proinflammatory cytokine responses (Karpala et al., 2011; Liniger et al., 2012). The tree shrew lineage also appears to have lost RIG-I, and pathogen pressures on tree shrew MDA5 and LGP2 have selected for the ability to detect the RIG-I agonist Sendai virus (SeV) (Xu et al., 2016). Chicken MDA5, unlike mammalian MDA5, preferentially recognizes short dsRNA (Hayashi et al., 2014), and like human MDA5 it can also be stimulated with long polyinosinic-polycytidylic acid (poly (I:C) (Barber et al., 2010). It is currently unknown if duck MDA5 has a dsRNA length preference. Chicken MDA5 also appears to have undergone positive selection, and is able to recognize RNA from Newcastle Disease virus (NDV) (Xu et al., 2019). Indeed, when these mutations were introduced into human MDA5, a glutamic acid to a leucine at position 633, the mutant was able to bind NDV RNA. Duck MDA5 has proline at residue 633 (Barber et al., 2010), and thus is not expected to detect NDV RNA.

MDA5 is most highly expressed in the trachea followed by the ileum, duodenum, crop, rectum, and colon in Muscovy ducks (Wei et al., 2014), like basal expression of RIG-I (Cheng et al., 2015a). In healthy adult chickens, MDA5 was most highly expressed in the spleen, followed by the thymus and trachea (Bush et al., 2018). Chicken MDA5 is strongly upregulated in lung, spleen and brain in H5N1 infected birds (Karpala et al., 2011). Duck MDA5 is upregulated in response to IAV infection at 1 DPI in the lung, spleen, and brain, and returns to normal levels at 3 DPI (Wei et al., 2014; Fleming-Canepa et al., 2019). MDA5 was also slightly upregulated in lungs of Pekin ducks infected with LPAI, but not significantly upregulated in intestines of the same cohort of ducks (Fleming-Canepa et al., 2019).

LGP2

LGP2 is induced in humans during influenza infection. LGP2 seems to function as both a positive and negative regulator of RIG-I and MDA5. This contrary effect on IFN signaling seems to be dose dependent as smaller amounts of LGP2 help increase MDA5 and RIG-I activation while over-expression of LGP2 inhibits it (Rothenfusser et al., 2005; Satoh et al., 2010). In mice infected with IAV, LGP2 attenuates the IFN response, perhaps in an effort to control damaging inflammatory responses (Malur et al., 2012). Recently LGP2 has also been implicated in inhibition of Dicer dependent processing of dsRNA, thus inhibiting RNAi (van der Veen et al., 2018). Muscovy ducks infected with HPAI H5N1 had upregulation of duck LPG2 (duLGP2) in the spleen at 1 DPI (Jiao et al., 2015). In the lung and brain, duLGP2 was upregulated on both 1 and 2 DPI suggesting that duLGP2 is involved in the early response to IAV. This is the same expression pattern seen in geese infected with this strain of H5N1 (Wei L. et al., 2016). No studies have been published to date on duLGP2 interactions with RIG-I during IAV infection. However, duLGP2 was important during duck enteritis virus (DEV) infection through interactions with MDA5 (Huo et al., 2019). Overexpression of chicken LGP2 (chLGP2) reduced IFN signaling in IAV infected cells, however silencing of the LGP2 gene in chicken cells also decreased IFN-β production, suggesting chLGP2 is important for MDA5 signal enhancement at low expression levels (Liniger et al., 2012). It is unknown if duLGP2 augments signaling with duck RIG-I or MDA5.

TRIM25

Tripartite motif protein 25 (TRIM25) can both augment IFN signaling (Gack et al., 2007) and directly restrict virus in mammals (Meyerson et al., 2017). TRIM25 is known to stabilize RIG-I CARD domain interaction with MAVS CARD domains and increase IFN production during an infection (Gack et al., 2007). The CARD domains of RIG-I are exposed when RIG-I recognizes viral RNA, at which point TRIM25 binds to RIG-I CARD domains using its C-terminal PRY-SPRY domain.

Using the E3 ligase activity of its RING domain, TRIM25 polyubiquitinates RIG-I, attaching K63-linked ubiquitin chains to lysine residues on RIG-I. Stabilization of the RIG-I CARD domain tetramer allows it to nucleate MAVS filament formation (Peisley et al., 2014). TRIM25 can also physically block vRNA transcription in the nucleus by binding to the vRNP complex (Meyerson et al., 2017). Whether duck TRIM25 has the ability to restrict viral RNA transcription not yet been examined.

Duck TRIM25 performs much the same function as human TRIM25 in RIG-I stabilization. Human TRIM25 ubiquitinates lysine 172 of human RIG-I CARD domains, but this lysine is not conserved in ducks. Instead, duck TRIM25 ubiquitinates K167 and K193 (Miranzo-Navarro and Magor, 2014). Mutation of either lysine site alone in the duck did not alter ubiquitination patterns of the CARD domains, however mutation of both sites abrogated covalently attached ubiquitin. Interestingly, duck TRIM25 in our transfection experiments could still activate these double mutants, suggesting unanchored ubiquitin could also stabilize RIG-I in the duck. Chicken TRIM25 augments IFN signaling, however the mechanism is unclear in the absence of RIG-I (Rajsbaum et al., 2012). In human cells, a long non-coding RNA (lncRNA) Lnczc3h7a also contributes to stabilizing the interaction between TRIM25 and RIG-I CARD domains (Lin et al., 2019). Recently, duck lncRNA were analyzed during HPAI and LPAI infection to determine which were differentially expressed and potentially involved in influenza A control (Lu et al., 2019). This study did not assess whether lnczc3h7a is differentially expressed, nor is it known if duck lnczc3h7a can function in the same manner, but this augmentation by lncRNAs may well be conserved.

In healthy chickens, TRIM25 is most highly expressed in the lung, spleen, and thymus and is upregulated in response to NDV in the spleen, thymus, and bursa (Feng et al., 2015). To date, we are unaware of studies looking at TRIM25 basal tissue expression in duck, however we showed TRIM25 is upregulated in the lung of HPAI infected ducks and slightly upregulated in lung of LPAI infected ducks at 1 DPI (Fleming-Canepa et al., 2019).

MAVS

MAVS protein is an adaptor protein that acts as a signaling amplifier during viral infection through interactions with both RIG-I and MDA5 (**Figure 1**). MAVS forms "prion-like" aggregates on the surface of the mitochondria when nucleated by tetramers of CARD domains of RIG-I or MDA5 (Kawai et al., 2005; Hou et al., 2011). The 2CARD domains of RIG-I form a helical tetrameric structure offset by 1 unit, and this helical assembly recruits MAVS CARD monomers (Wu et al., 2014). The helical assembly of tetrameric RIG-I and elongation of MAVS filaments is necessary for signal transduction by MAVS. Although ducks have very different amino acid sequences within these CARD domains compared to mammals, we showed the helical assembly of d2CARD with MAVS leads to signal activation as well (Wu et al., 2014). Filamentous MAVs then recruits tumor necrosis factor receptor associated factor 3 (TRAF3), which acts as an adaptor protein to phosphorylate TANK-binding kinase 1 (TBK1) and inhibitor of nuclear factor-κB (IκB) kinase (IKK) (Fitzgerald et al., 2003; Liu S. et al., 2015). From there transcription factors such as interferon regulatory factor 3 or 7 (IRF3/IRF7) are activated to induce IFN production.

Duck MAVS expression in healthy tissues varied depending on the age of the ducks tested. In 3-week old Cherry Valley ducks, MAVS expression was highest in the pancreas, liver and heart (Li N. et al., 2016), while in 2-month-old Cherry Valley ducks, tissue expression was more ubiquitous with slightly higher expression seen in the trachea and heart (Li H. et al., 2016). MAVS basal expression in adult chickens is also more ubiquitous, with only slighter higher expression seen in the spleen, heart, and thymus (Bush et al., 2018). The human protein atlas shows that human MAVS is expressed in almost all tissues, but curiously has the lowest expression in innate immune cells such as dendritic cells, monocytes, T-cells and B-cells (Uhlen et al., 2015). Pekin duck MAVS is upregulated 1 DPI in both HPAI and LPAI infection in lungs, however no MAVS upregulation was seen in ileum of LPAI infected ducks (Fleming-Canepa et al., 2019).

TBK1

TBK1 activates IFN-β production by phosphorylating IRF3 allowing it to dimerize and translocate to the nucleus and initiate type I IFN production (Fitzgerald et al., 2003; Liu S. et al., 2015) (**Figure 1**). In humans TBK1 (huTBK1) expression is highest in brain tissues, adrenal glands, lungs, and the upper digestive tract (Uhlen et al., 2015). Chickens express TBK1 highest in spleen, lung, and thymus (Wang et al., 2017). This contrasts with 1-month old Cherry Valley ducks, where the highest expression was seen in the liver, heart, and duodenum (Hua et al., 2018). Very little expression was seen in healthy lungs, spleen, or bursa of these ducks. Duck TBK1 (duTBK1) was shown to function similarly to huTBK1 in that overexpression was able to activate IFN-β, NF-κB, and IRF1 promoter activity in duck embryonic fibroblast (DEF) cells. Silencing of endogenous duTBK1 in DEF cells also significantly reduced IFN-β promoter activity in DEF cells. As basal tissue expression of many duck PRR and downstream signaling components seems to favor having reduced expression of these proteins in immune relevant sites, we suggest that this could be another level of immune regulation that is protective to the duck. Experimental dysregulation of basal tissue expression of proteins such as TBK1 and IRF7 could be done to investigate this question.

TRAF3

TRAF3 operates downstream of both TLRs as well as RLRs to aid in signal transduction and amplification (Hacker et al., 2006) (**Figure 1**). In the RIG-I signaling pathway, TRAF3 acts as an adaptor downstream of MAVS, by recruiting TBK1 and IKKε to phosphorylate the transcription factor IRF3 (Guo and Cheng, 2007). TRAF3 is most highly expressed in lung, spleen, and thymus of 2-week-old chickens (Yang et al., 2015). Duck TRAF3 (duTRAF3) however, has a uniform expression pattern with only slightly higher amounts of TRAF3 expression seen in the brain, and the lowest levels in the lung (Wei et al., 2018). In chicken embryonic fibroblasts (CEF) cells, TRAF3 (chTRAF3) is upregulated in response to poly (I:C) stimulation, NDV infection and poly dA-dT, suggesting it is important in both DNA and RNA viral infections (Yang et al., 2015). Similarly, duTRAF3

is also upregulated in DEF cells stimulated with poly (I:C) and the authors also found that overexpression of duTRAF3 could control both IAV and duck Tembusu virus replication (Wei et al., 2018).

Curiously, a truncated version of duTRAF3 was also found, named duTRAF3-S (splice isoform duck TRAF3) (Wei et al., 2018). This splice variant is missing key N-terminal catalytic domains but can still bind to both TBK1 and MAVS with its C-terminal TRAF domain. DuTRAF3-S can interact with duTRAF3 but not MAVS, thus decreasing IFN-β production. After poly (I:C) stimulation, DEF cells express more duTRAF3 until 9 HPI, at which point duTRAF3 mRNA expression begins to decrease and duTRAF3-S mRNA expression begins to increase. This splice isoform may act to dampen IFN signaling in the later time points of infection to reduce damage from inflammation. In summary, duTRAF3 is most highly expressed in the brain in healthy ducks, while chickens express more in the lung, spleen, and thymus.

IRF7

IRF3 is a known important mediator of the type I interferon system in mammals. IRF3 is ubiquitously expressed, slow to degrade and a potent transcriptional activator of Type I IFN production in mammals (Honda and Taniguchi, 2006). Birds appear to be missing IRF3, however, they do have IRF7 (Cormican et al., 2009; Huang et al., 2010). Avian IRF7 is structurally like IRF3, suggesting that it may play a similar role to that of IRF3 in mammals. Recent bioinformatics analysis has confirmed that chicken IRF7 clusters more closely to IRF3 of lower vertebrates, yet is located in a region with high synteny to mammalian IRF7 (Cheng et al., 2019b).

IRF3, rather than IRF7, is considered more important for the initial response to viral infection. In mammals IRF3 is constitutively expressed in most tissues and seems to have a long half-life (Prakash and Levy, 2006; Hiscott, 2007). Activation of IRF3 results in increased type I IFN signaling and an eventual increase in transcription of IRF7, which has a very short half-life, comparatively. IRF7, in turn, amplifies both Type I and Type III IFN signaling (Sato et al., 1998). In mice, knockdown of IRF3 is not detrimental to the IFN response to IAV, however knockdown of IRF7 leaves mice much more susceptible to infection and a double knockout of both transcription factors renders mice unable to produce IFN-α or IFN-β (Hatesuer et al., 2017). Humans who have mutations in IRF7 are more susceptible to life threatening infections by IAV (Ciancanelli et al., 2015). There is very little expression of duck IRF7 (duIRF7) in the lung of uninfected ducks, and greater expression seen in the liver and intestine (Chen et al., 2019). Chicken IRF7 (chIRF7) is most highly expressed in the spleen and lung of healthy chickens (Cheng et al., 2019b).

Recent research has focused on the role of IRF7 in inhibition of IAV through IFN mediated responses in chickens and ducks. Chicken IRF7 (chIRF7) is involved in antiviral responses and plays analogous roles to that of mammalian IRF3. Recent studies have found that chIRF7 can be induced to translocate across the nucleus downstream of both chMAVS and chicken stimulator of interferon genes (chSTING), and chIRF7 dimerizes following chTBK1 activation, allowing it to increase IFN-β signaling (Wang et al., 2019). Initial experiments investigating function found that overexpression of chIRF7 increased IFN-β expression (Kim and Zhou, 2015). However, their knockdown of chIRF7 did not significantly change IFN-β expression during poly (I:C) stimulation suggesting other transcription factors may be involved. Contradictory results were published in 2019 showing chirf7$^{-/-}$ DF-1 cells were unable to produce IFN-β, even when transfected with MAVS or STING (Cheng et al., 2019b). DuIRF7 upregulates type I IFNs but does not affect type II IFN expression (Chen et al., 2019). We showed that duIRF7 increases IFN-β signaling when overexpressed in DF-1 cells (Xiao et al., 2018). We also observed duIRF7 translocate to the nucleus upon stimulation with constitutively active RIG-I 2CARD. When chIRF7 is overexpressed in DF-1 cells, it caused increased cell death and resulted in higher levels of viral replication (Kim and Zhou, 2018). With transfection of mCherry-IRF7 (Xiao et al., 2018), we also observed increased cell death.

IRF7 can control viral replication in ducks. A recent study demonstrated that duIRF7 can control the positive sense RNA virus, duck Tembusu virus in DEF cells (Chen et al., 2019). No studies to date have examined whether duIRF7 controls IAV, or if it increases viral replication, as seen in DF-1 cells. This may be an interesting avenue of study, as Kim and Zhou (2018) suggest that chIRF7 could be a target of IAV.

STING

Stimulator of interferon gene (STING) is a protein on which many PRR pathways converge in order to increase NF-κB and IFN signaling downstream of pathogen pattern recognition (**Figure 1**). It was initially discovered as an adaptor molecule in the cyclic GMP-AMP synthase (cGAS) signaling pathway, which detects viral DNA and subsequently drives the induction of type I IFNs and proinflammatory cytokines (Ishikawa et al., 2009). STING also interacts with both RIG-I and MAVS in mammalian cells and is involved with sensing of RNA viruses (Zhong et al., 2008; Castanier et al., 2010). STING is found on the endoplasmic reticulum and can be closely associated to MAVS on the mitochondrial outer membrane (Zhong et al., 2008; Ishikawa et al., 2009). Acting as a scaffolding protein between TBK1 and IRF3, STING aids in IRF3 phosphorylation and type I IFN induction (Zhong et al., 2008; Tanaka and Chen, 2012). IAV interferes with STING through its hemagglutinin fusion peptide, effectively preventing STING dimerization and interactions with TBK1 (Holm et al., 2016). In addition, independently of RIG-I or TLR detection, STING also detects RNA viral membrane fusion events and potentiates the IFN response during viral infection (Holm et al., 2012).

Duck STING (DuSTING) shares 43 and 71% identity to human and chicken STING (chSTING), respectively (Cheng et al., 2019a). DuSTING is most highly expressed in the glandular stomach, followed by the trachea, lung, small intestine, spleen, kidney, and bursa (Cheng et al., 2019a). ChSTING is most highly expressed in the thymus, bursa, spleen, lung, and intestine of uninfected chickens (Ran et al., 2018). As chSTING was not analyzed in the glandular stomach or trachea, it is not possible compare expression to ducks. However, it is noteworthy that in ducks, STING is more abundant in the lung than the bursa and spleen. If duSTING is orthologous to mammalian STING, it may react to IAV fusion quicker in these tissues although it is not

known if duSTING can detect viral fusion. Human STING shows low tissue specific expression, but has slightly higher mRNA expression in tonsils, lymph nodes, and lung (Uhlen et al., 2015).

Human STING increases IFN-β signaling when overexpressed in 293 T cells (Ishikawa et al., 2009). MEF cells were shown to require STING but not cGAS to produce IFN after infection with two RNA viruses, NDV and SeV. Similarly, duSTING drastically increased IFN-β promoter activation when overexpressed in DEF cells. However, when the cells were stimulated with poly (I:C), STING was not required to potentiate the IFN response (Holm et al., 2016). DuSTING is highly upregulated in both spleen and lung in ducks infected with a LPAI H9N2. DuSTING was most highly upregulated on day 2 in both these tissues. In lungs, duSTING was only upregulated on day 2, with day 1 showing no significant increase when compared to mock infected birds (Cheng et al., 2019a). This may be because a LPAI strain of virus was used. It would be interesting to look at STING regulation in these tissues during HPAI infection.

TOLL-LIKE RECEPTOR PATHWAY

TLRs are important pattern recognition receptors that induce innate immune responses to viral, bacterial, fungal and parasitic pathogens (Kawai and Akira, 2010). Humans have 10 TLRs (TLR1-10) as do birds, however the TLRs that have been classified in birds are different, as reviewed by several groups (Boyd et al., 2007; Temperley et al., 2008; Brownlie and Allan, 2011; Chen et al., 2013; Keestra et al., 2013). For example, TLR1 in birds has been duplicated so that birds express TLR1a and TLR1b. Similarly, TLR2 has two paralogous genes, *tlr2a* and *tlr2b*. Other homologous TLRs expressed by birds include TLR3, TLR4, TLR5, and TLR7, which leaves TLR8, TLR9, and TLR10 currently unaccounted for in avian species. Birds also have two TLRs which are not found in mammals but have been classified in lower vertebrates: TLR15 and TLR21. TLR15 is upregulated in response to bacterial pathogens in chickens (Nerren et al., 2010), and recognizes a yeast-derived agonist (Boyd et al., 2012) and diacylated lipopeptide from mycoplasma (Oven et al., 2013). TLR21 functions analogously to TLR9 in humans in that it recognizes CpG oligodeoxynucleotides (CpG ODN) in both duck (Cheng D. et al., 2019) and chicken (Brownlie et al., 2009).

TLRs can be expressed both extra and intracellularly, with the cell surface TLRs being more adept at detecting extracellular pathogens (TLR1, 2, 4, 5, and 6) (Hopkins and Sriskandan, 2005). Likewise, TLRs that are in endosomes, or in other intracellular compartments, are more specialized in detecting intracellular pathogens, such as viruses (TLR3, 7, 8, and 9). Specific TLRs, such as TLR3, TLR7, and TLR8 recognize viral RNA and play important roles in the defense against IAV in mammals (Alexopoulou et al., 2001).

PAMPs are detected through the TLR ectodomain with leucine rich repeats (LRR) and signal downstream to produce IFNs and other cytokines through their cytoplasmic Toll/IL-1 receptor (TIR) domain (Botos et al., 2011). TLRs are activated and different adaptor proteins are recruited to amplify the signal. TIR-domain-containing adapter-inducing interferon-β (TRIF) dependent pathways induce type I IFN production through TBK1 and IRF3 activation (Sato et al., 2003; Yamamoto et al., 2003). Myeloid differentiation primary response 88 (MyD88) dependent pathways induce NF-κB proinflammatory gene expression through recruitment of TRAF6 and eventual activation of the IKK signaling complex (Hemmi et al., 2002; Muroi and Tanamoto, 2008).

Induction of TLR signaling increases IFN production and cytokine signaling in both mammalian and avian cells. As such, treatment of cells with TLR specific ligands such as poly (I:C), lipopolysaccharide (LPS) and CpG ODN can reduce IAV replication in both mammals (Cluff et al., 2005; Shinya et al., 2011) and chickens (St. Paul et al., 2012; Barjesteh et al., 2014). TLRs can also act synergistically to produce proinflammatory responses. In chicken monocytes, stimulating with the TLR3 ligand poly (I:C) resulted in an increase in mRNA of type I IFNS (He et al., 2012). Co-stimulation of these chicken monocytes with the TLR21 ligand CpG-ODN and poly (I:C) resulted in an even greater increase of proinflammatory cytokines than cells stimulated with a single ligand and biased the cells to a Th1 type response. Since only TLR3 and 7 directly detect IAV during infection in birds, we will focus on these TLRs in the next two sections.

TLR3

TLR3 is an endosomal TLR that recognizes dsRNA or replicating viral intermediates and activates NF-κB signaling in a TRIF dependent signaling pathway (Alexopoulou et al., 2001) (**Figure 1**). In humans, TLR3 is predominantly expressed in the placenta, followed by smaller but still significant amounts in the small intestine and lower amounts in most other tissues (Uhlen et al., 2015). It is also constitutively expressed in bronchial and alveolar epithelial cells (Guillot et al., 2005). Infection of the human cell line A549 (alveolar epithelial cell line) with IAV resulted in an upregulation of TLR3 (Wu et al., 2015). TLR3 stimulation during influenza infection resulted in activation of IRF3 and increased type III IFN production. When TLR3 knockout mice were infected with influenza they had a surprising survival advantage over wildtype mice, despite having higher viral titres in their lungs (Le Goffic et al., 2006), highlighting the complex role of this PRR in influenza restriction.

Tissue expression of TLR3 differs between ducks and chickens (**Figure 2B**). In uninfected tissues, Pekin duck TLR3 is expressed highest in the trachea with lower expression seen in the digestive tissues and the lung (Zhang M. et al., 2015). Muscovy ducks, which are more susceptible to influenza virus infection than Pekin or mallard ducks (Pantin-Jackwood et al., 2013) show higher expression of TLR3 in the trachea, spleen, pancreas, lung, and digestive tissues (Jiao et al., 2012). Thus, Muscovy ducks show high basal expression of TLR3 in many tissues, while Pekin ducks had high expression only in trachea. In chickens, basal TLR3 expression is highest in intestine, liver, and kidney (Iqbal et al., 2005). TLR3 was constitutively expressed in chicken heterophils (Kogut et al., 2005).

After infection with HPAI virus, Muscovy duck TLR3 was upregulated at 24 HPI in the lung and brain, with sustained expression in the brain (even though this is a non-fatal infection

in Muscovy ducks) (Jiao et al., 2012). There was no increased expression in the spleen. In contrast, transcriptomic data from Shaoxin mallard ducks infected with a HPAI H5N1 show increased TLR3 expression in the lungs, peaking on day 2 of infection (Huang et al., 2013). This discrepancy between the Muscovy duck and Shaoxin mallard TLR3 expression data may be due to the strains of virus used in the infection (DK212 vs. DK49; both H5N1) but not age of the birds as both experiments used 4-week old ducks. Chickens upregulated TLR3 in the lung during HPAI H5N1 infection when replicating virus was still present in lung tissues (Ranaware et al., 2016). In reovirus infected ducks, TLR3 expression peaked at 72 HPI in the lung, while spleen and bursa showed a sustained response from 24 to 48 h (Zhang M. et al., 2015). These results are of interest as Reovirus infection in Muscovy duck can cause mortality in 20–40% of infected animals (Malkinson et al., 1981; Wozniakowski et al., 2014).

TRIF

TRIF is the adaptor molecule downstream of TLR3 and TLR4 and provides a signaling platform to recruit other adaptor proteins and increase type I IFNs and proinflammatory cytokine expression (**Figure 1**). Similar to humans (Yamamoto et al., 2003), in uninfected tissues, ducks express TRIF most highly in the pancreas and spleen (Wei X. et al., 2016) (**Figure 2B**). Chicken TRIF expression was found to be highest in the cecum, heart, liver, spleen, and kidney (Wheaton et al., 2007). Expression of duck TRIF peaks at 12 h after treatment with poly (I:C), however, it peaks much later at 36 h post infection with IAV (Wei X. et al., 2016), likely due to viral suppression of IFN signaling pathways in infected cells.

TLR7

Human TLR7 produces a robust type I IFN response upon detection of IAV or other ssRNA viruses using the MyD88-dependent pathway (Diebold et al., 2004; Lund et al., 2004). TLR7 is highly expressed by murine plasmacytoid dendritic cells (pDCs) and is located in endosomal compartments where it can detect incoming viral RNA (Diebold et al., 2004), and produce high levels of IFN-α. RNA from live and inactivated influenza virus can be detected by TLR7 in the endosome of pDCs, provided the hemagglutinin remains intact for receptor-mediated viral entry (Diebold et al., 2004) TLR7 detection is thus known to induce IFN-α, and proinflammatory cytokines (**Figure 1**). Suggesting that the role of TLR7 and RIG-I signaling is complicated in influenza infection, *Tlr7*$^{-/-}$*Mavs*$^{-/-}$ knockout mice succumb quickly to a lethal influenza infection as expected, however infection with a low viral dose revealed that proinflammatory signaling promoted viral replication by recruiting susceptible monocytes (Pang et al., 2013). Oddly, humans have enhanced tissue expression of TLR7 in the brain, with lower expression in mucosal tissues (Uhlen et al., 2015).

Tissue expression of TLR7 is notably different between healthy ducks and chickens (**Figure 2B**). Duck TLR7 is expressed the highest in spleen, bursa, and lung (MacDonald et al., 2008; Kannaki et al., 2018). In chickens, basal TLR7 expression is highest in spleen, bursa, and intestine with very little expression in the lung (Iqbal et al., 2005; Philbin et al., 2005), initially suggesting that this distribution may play a role in chicken susceptibility to HPAI strains that replicate in the lungs. However, the chicken macrophage cell line HD11 expresses high levels of TLR7 (Philbin et al., 2005), and both primary macrophages and heterophils constitutively express TLR7 in other studies (Kogut et al., 2005). The chicken atlas on the BioGPS server agrees with the previous studies in that TLR7 expression is limited in the lung, and higher in tissues such as the spleen, bursa, and immune cells (Bush et al., 2018). It is however worth noting that TLR7 basal expression in chickens is slightly variable depending on the breed and age of chicken sampled. Stimulation using TLR7 agonists decreased viral replication in chicken macrophages (Stewart et al., 2012; Barjesteh et al., 2014; Abdul-Cader et al., 2018), indicating TLR7 can induce IFNs in those cell types. Thus, chicken strains may vary with respect to TLR7 expression. Ducks infected with HPAI upregulate TLR7 most highly in their lungs 2 DPI while chickens infected with the same virus had only a slight increase in expression at 1 DPI (Cornelissen et al., 2013). In contrast, ducks infected with a LPAI H7N9 had only marginal upregulation of TLR7 In their lungs 0.8 DPI, while chickens had a significant increase in this expression 0.8 DPI (Cornelissen et al., 2012).

MyD88

MyD88 conveys the signal downstream of most of the TLRs, to induce an inflammatory response upon detection of pathogens (**Figure 1**). MyD88 signaling was found to be important for protecting mice during primary influenza infection, as MyD88$^{-/-}$ knockout mice were more susceptible (Seo et al., 2010). MyD88 may also be an important factor in initiating damaging cytokine storms in the host, since there was a significant reduction in proinflammatory cytokines and activated macrophages and neutrophils in the lungs of MyD88$^{-/-}$ mice, but not TRIF$^{-/-}$ mice, following IAV infection (Teijaro et al., 2014). Ducks have two isoforms of the *myd88* gene that have been characterized, named DuMyD88-X1 and DuMyD88-X2 (Cheng et al., 2015b). DuMyD88-X2 is a truncated version that encodes a premature stop codon and produces a protein with an interruption in the TIR signaling domain. DuMyD88-X1 is highly expressed in uninfected ducks in all immune relevant tissues including the lung, intestine, and bursa, but it showed the strongest expression in the spleen (**Figure 2B**). DuMyD88-X2 was expressed in these same tissues but to a much lower extent than the X1 isoform. Both isoforms of MyD88 could activate the IL-6 promoter and induce NF-κB activity in duck cells. In ducks challenged with NDV, the X1 isoform was upregulated in liver and spleen. Neither isoform was as highly expressed in the lung during NDV infection, and no studies have looked at the expression of these genes during influenza infection. Three isoforms of MyD88 have been found in chickens (named MyD88-1, 2, and 3) (Qiu et al., 2008). Chicken MyD88 (chMyD88) is the largest of the isoforms, and is ubiquitously expressed, which agrees with previous research on chMyD88 expression although it is of note that these studies demonstrated slightly more chMyD88 expression in the thymus, liver, and spleen than in other tissues tested (Wheaton et al., 2007). ChMyD88 is not significantly upregulated in DF-1 cells infected with influenza (Barber et al., 2013). Upregulation in influenza-infected chicken

tissues has not been explored, but MyD88 is upregulated by LPS treatment (Wheaton et al., 2007). As MyD88 plays a role in immune system derived damage during influenza infection in mammals, it would be interesting to know if chMyD88 activation is significantly different from the duck.

NLR RECEPTORS—THE NLRP3 INFLAMMASOME

NLRP3

The NOD-like receptor family pyrin domain containing 3 (NLRP3) can form multi protein complex inflammasomes, which possess autocatalytic activity. This activity can activate caspase-1 and induce the production of proinflammatory cytokines IL-1β and IL-18 (**Figure 1**). NLRP3 inflammasome induction can occur in immune cells such as macrophages (Pirhonen et al., 2001) and dendritic cells (Fernandez et al., 2016) and as well in other cell types such as fibroblasts and epithelial cells (Allen et al., 2009; Pothlichet et al., 2013). Deletion of NLRP3 in mice causes a decrease in immune cell recruitment to the site of infection and poor outcomes when infected with influenza (Allen et al., 2009; Thomas et al., 2009).

Tissue expression of NLRP3 differs between ducks and chickens (**Figure 2C**). NLRP3 is fairly ubiquitously expressed in healthy chicken tissues but most highly expressed in chicken trachea and lung (Ye et al., 2015). Duck NLRP3, however, is most highly expressed in the pancreas with very low expression in the lung and slightly higher expression in the trachea (Li et al., 2018). This expression profile is of interest as NLRP3 inflammasome activation has been associated with contributing to cytokine storms and severe pathology from influenza infection (Teijaro et al., 2014). We are unaware of studies detailing the NLRP3 inflammasome response to influenza infection in either chicken or duck.

IRF1

IRF1 is known to be an activator of IFNs though several mechanisms, but one of importance is its regulation of the NLRP3 inflammasome (Kuriakose et al., 2018) (**Figure 1**). It is thought that by regulating the NLRP3 inflammasome, IRF1 contributes to apoptosis and necroptosis during influenza infection. Kuchipudi et al. (2012) suggest that duck cells are more likely to become apoptotic when infected with IAV than chicken cells. Indeed, DEF cells infected with HPAI strains that are known to cause severe symptoms in infected ducks had decreased apoptosis (Kuchipudi et al., 2012). Thus, IRF1 as a regulator of early apoptotic response is an interesting candidate to study in ducks. Human IRF1 is expressed highest in the spleen and the liver (Uhlen et al., 2015). Duck IRF1 (duIRF1) is most highly expressed in liver and spleen, followed by the pancreas, and digestive tissues such as the stomach and duodenum. Interestingly, it is expressed in very low levels in the lung and trachea (Qian et al., 2018) (**Figure 2C**). The chicken atlas on the BioGPS server indicates that chicken IRF1 (chIRF1) expression in healthy adult birds is highest in the lung, spleen, and thymus (Bush et al., 2018).

Overexpression of chIRF1 in DF-1 cells caused a significant increase of IFN-β, Mx, and MDA5 mRNA (Liu Y. et al., 2018). chIRF1 mRNA also substantially increased 12 HPI after infection with either IAV or NDV. These transcripts rapidly dropped back down to basal levels after 12 h. Poly (I:C) stimulation of duck fibroblasts resulted in duIRF1 transcripts peaking at 12 HPI and then decreasing, as in chicken cells. However, when these cells were infected with H5N1 the duIRF1 mRNA began to increase at 12 HPI and continued to increase until 48 HPI. The delay in the duck response may be due to strain differences between viruses used (Qian et al., 2018) as the chIRF1 study used A/Chicken/Shanghai/010/2008 (H9N2) while the duIRF1 study used A/Duck/Hubei/hangmei01/2006 (H5N1). DuIRF1 interacts with MyD88 to increase IFN-β independently of IRF7, and overexpression of duIRF1 not only upregulated Type I IFNs but also Type III IFN (IFN-λ) (Qian et al., 2018). When ducks were infected with H6N2, duIRF1 transcripts peaked at 36 HPI, rather late in infection compared to other ISGs or IFNs mentioned in this article. As duIRF1 does not signal downstream of RIG-I, it could be used as a secondary pathway to limit viral replication. Overexpression of duIRF1 also limited H9N6 and H5N1 viral replication.

INTERFERON RESPONSES AND ISGS

Type I IFNs

Type I interferons include IFN-α and IFN-β, both which are present in birds (Santhakumar et al., 2017). Airway epithelium, macrophages, and pDC are responsible for most of the type I IFNs produced during viral infection (Onoguchi et al., 2007; Khaitov et al., 2009; Crotta et al., 2013). Plasmacytoid dendritic cells are known to produce much of the initial IFN-α (Ito et al., 2005; Liu, 2005), and it is thought that the autocrine action of IFN-α on the pDCs upregulates antiviral factors such as Mx1 and thus protects against influenza infection (Cella et al., 1999). An early IFN response generally provides more positive outcomes in infection, and studies have also implicated type I IFN responses as a factor that can reduce pro-inflammatory cytokine release and thus limit damage (Billiau, 2006; Guarda et al., 2011; Arimori et al., 2013).

Both transcriptomic and qPCR studies have demonstrated that ducks have a robust but short response of type I IFNs in response to HPAI (Cagle et al., 2011; Vanderven et al., 2012; Saito et al., 2018). Transcriptomic data demonstrated that lungs of ducks infected with a HPAI H5N1 strain had an increase in *IFNA* expression days 1 and 2 DPI (Huang et al., 2013). While IFNs are most strongly upregulated within the first 24 h, it should be noted that many ISGs have a sustained response for up to 3 DPI (Huang et al., 2013; Smith et al., 2015). Ducks infected with HPAI H5N1 strains A/goose/Guangdong/16568/2016 (GS16568), and A/duck/Guangdong/16873/2016 (DK16873) showed sustained responses of type I IFNs post infection. However, the time points used in these experiments were 12 HPI and 2 DPI (Wu et al., 2019). While these highly pathogenic strains of flu could be eliciting sustained responses, other strains of H5N1 had the peak of IFN upregulation at 1 DPI (Saito et al., 2018). LPAI

induces a relatively weak IFN response in ileum of infected ducks (Vanderven et al., 2012).

In ducks infected with HPAI H5N1 strains VN1203 and D4AT, we found that IFN-α and IFN-β were most upregulated 1 DPI in lungs and spleens of infected birds (Saito et al., 2018). The spleen had a greater increase in IFN-α transcripts compared to the lung, while lung showed higher upregulation of IFN-β. This may reflect the relative contribution of different PRRs in these tissues; while TLRs are largely responsible for IFN-α, IFN-β expression is largely RIG-I dependent (Opitz et al., 2007). By day 2 the IFN response had been reduced to mock infection levels. When testing the expression of IFN-α in primary avian cells infected with either H5N1 or H5N9, it was highest in duck cells at 12 and 24 HPI (Jiang et al., 2011). In chicken and turkey cells, IFN-α was most highly expressed at 24 HPI.

Pre-treatment with IFN-α protects duck cells, but not adult ducks from IAV infection. DEF cells treated with IFN-α show a reduced viral load as well as induction of many ISGs (Gao et al., 2018b). Interestingly, pre-treatment of primary chicken lung cells and duck fibroblasts with IFN-α before infection with IAV reduced IFN-α production in both these cell types (Jiang et al., 2011). The protective effects of IFN-α seem to be age dependent in the duck. When looking at survival rates of 2 days vs. 3 weeks old ducklings treated with rIFN-α before infection of HPAI H5N1, the treatment with IFN benefited the 2 days old ducklings but not the 3 weeks old ducks (Gao et al., 2018b). The rIFN-α dose may have been insufficient to protect the older ducks, or alternatively IFN-α is not protective. In contrast, 7 and 33-day old chickens treated with rIFN-α before exposure to a chicken-isolate H9N3 were both found to be protected (Meng et al., 2011). These results are of interest, as generally younger ducks are more susceptible to IAV infection, and protection correlates with onset of RIG-I expression (Londt et al., 2010; Pantin-Jackwood et al., 2012). The DK383 H5N1 virus used, which is lethal in ducks (Gao et al., 2018b), may impair RIG-I signaling, and IFN-α alone is not sufficient to protect the older ducks. Similarly, IFNB knockout mice are much more sensitive to influenza, suggesting IFN-α cannot fully compensate (Koerner et al., 2007). These results seem to support the hypothesis that an early and quick response is more beneficial to the duck than a sustained type I IFN response.

Type II Interferons

IFN-γ is classified as a type II IFN and is secreted by NK cells, $CD8^+$ lymphocytes and $CD4^+$ T helper cells (Schroder et al., 2004). While IFN-γ has been found in some studies to be protective against influenza (Weiss et al., 2010), other researchers have shown that by knocking out the genes or knocking down gene expression in mice, absence of IFN-γ protected the mice from severe infection with pandemic H1N1 (Califano et al., 2018). Similarly, other studies in mice have shown that IFN-γ negatively regulates the survival of CD8+ T cells during influenza infection and limits the number of influenza specific memory cells available during an infection (Prabhu et al., 2013).

CEFs treated with IFN-γ were more resistant to infection by H9N2 avian influenza virus and H1N1 human influenza virus. Stimulation with IFN-γ also increased IFN-α/β, and Mx transcripts in these cells (Yuk et al., 2016). Likewise, DEF cells treated with recombinant duck IFN-γ showed significant decreases in viral replication with a HPAI H5N1. Two-day old ducks were pre-treated with IFN-γ before being infected with DK383 IAV serotype H5N1. In these experiments 6/10 ducks that were pre-treated survived the infection at 10 DPI, while in PBS treated controls only 2/10 ducks survived (Gao et al., 2018a). As age played a factor in IFN-α pre-treatment reducing viral load in ducks, it would be worthwhile to repeat these experiments in older ducks. To our knowledge no studies have investigated whether duck IFN-γ influences the development of memory T cells during IAV infection.

Type III Interferons

Type III IFNs induce an antiviral state like that of type I IFNs but use different receptors for detection. Additionally, type III IFN receptors are expressed predominantly in airway epithelial cells and intestinal epithelia (Sommereyns et al., 2008), unlike type I IFN receptors, which are more ubiquitously expressed. Ducks and chickens express one kind of type III IFN (IFN-λ) (Karpala et al., 2008; Yao et al., 2014; Santhakumar et al., 2017) whereas other vertebrates produce one to four different type III IFNs, depending on the species (Kotenko et al., 2003; Chen et al., 2016).

Primary CEF and DEF cells both produce IFN-λ (chIFN-λ and duIFN-λ, respectively) in response to both poly (I:C) stimulation and infection with a mouse-adapted strain of H1N1 (Zhang Z. et al., 2015). Interestingly, DEF cells produce less IFN-λ transcripts when stimulated with poly (I:C) or infected with H1N1 than CEF cells. These same DEF cells also highly upregulate IFN-λ receptor transcripts at 36 HPI whereas the CEF cells highly express the receptor transcripts at 8 HPI and continue to do so until 36 HPI. A separate study found that chIFN-λ was unable to induce an antiviral state in the chicken fibroblast DF-1 cell line when infected with a HPAI H5N1, indeed the cells were not able to respond to recombinant chIFN-λ until they were transfected with the receptor (Reuter et al., 2014). This discrepancy may be due to the use of primary cells in one study and an immortalized cell line in the other. Immortalized cells often drastically change genotype and so the DF-1 cells may have stopped expressing the chIFN-λ receptor. High levels of the chIFN-λ receptor transcripts were found in the lung, trachea and intestine (Zhang Z. et al., 2015), suggesting that like chIFN-λ receptor expression is like that of humans. There is currently very little research on duIFN-λ, its receptor or antiviral activity, making this a promising candidate for future studies into IAV resistance in the duck.

OTHER ANTIVIRAL PROTEINS OF INTEREST

TRIM Proteins

TRIM proteins are a large family of intracellular proteins with diverse functions such as cell cycle regulation, autophagy, proteasomal degradation, development, and immunity which have been comprehensively reviewed (van Gent et al., 2018). Most interestingly, some of these proteins allow species-specific protection from viruses through viral restriction. One of the first TRIM proteins discovered, the alpha isoform of TRIM5

(TRIM5α) was found to restrict HIV in non-human primates, while the human ortholog was unsuccessful in restricting this virus (Stremlau et al., 2004; Sawyer et al., 2005). This highlights the evolutionary relationship these proteins have with pathogens and suggests that members of this protein family might be providing their host species a significant advantage.

A study from 2008 listed 38 TRIM genes in chicken, compared to human, rat, mouse, dog, and cow on their TRIMgene online database (Sardiello et al., 2008). Very few studies have been done on avian TRIM proteins. Avian TRIM25 has a specific role in the activation of RIG-I as discussed above in section TRIM25 (Rajsbaum et al., 2012; Miranzo-Navarro and Magor, 2014). A family of related TRIM genes was discovered in the avian MHC-B locus in both chicken (Ruby et al., 2005; Shiina et al., 2007) and duck (Blaine et al., 2015), with the MHC location suggesting this gene expansion may have arisen from pathogen pressures. The set of TRIM proteins in the MHC-B locus of birds all contain the B30.2/PRYSPRY C-terminal domain motif. Proteins containing this domain have recently expanded in TRIM protein evolution (Sardiello et al., 2008). The PRYSPRY domain is thought to be able to recognize specific amino acid sequences rather than peptide motifs, giving it pathogen specific activity (James et al., 2007; D'Cruz et al., 2013). Ducks also have an expanded butyrophilin gene family, proteins which also contain a B30.2/PRYSPRY domain (Huang et al., 2013).

Of the expanded TRIM genes in the duck MHC, TRIM27.1, and TRIM27-L were found to have antagonistic functions in the MAVS signaling pathway (Blaine et al., 2015). TRIM27-L significantly increased IFN-β signaling in a dose dependent manner while TRIM27.1 slightly decreased this same signaling in DF-1 cells. When co-expressed TRIM27-L activity overrode the inhibition of TRIM27.1. Curiously TRIM27-L appears to have been lost in Galliformes while being retained in Anseriformes, other birds and reptiles. As the Galliformes have also lost RIG-I it seems that either TRIM27-L expression was detrimental and thus lost in evolution or provided no benefit. Further, TRIM27.1 expression is higher in infected tissues than TRIM27-L. As the decrease in IFN-β was only slight, it could be that TRIM27.1 is playing another role in infection. TRIM27.1 may be upregulated to inhibit influenza without influencing cytokine signaling, as TRIM32 does in some human cell types (Fu et al., 2015). Of the chicken MHC-B TRIM genes, only TRIM39 has been cloned and tissue expression analyzed, but no function has been determined (Pan et al., 2011).

TRIM23 was identified as a differentially expressed gene in a microarray study from ducks infected with both HPAI and LPAI strains of IAV, as upregulated 5 DPI in LPAI but not HPAI infections (Kumar et al., 2017). TRIM23 is an ancient TRIM with well-conserved structural homology, and uses its ADP-ribosylation factor (ARF) domain to activate TBK1 through GTPase activity (Sparrer et al., 2017). TBK1 then activates selective autophagy, controlling viral replication. This is an interesting observation as LPAI virus can replicate in ducks for many days past initial infection, and the upregulation of TRIM23 suggests it is worth investigating whether it affects viral replication.

Finally, TRIM62 was identified as a retroviral restricting protein in chicken cells (Li et al., 2019), and until recently TRIM62 was only known to function in innate immune signaling augmentation in fish (Yang et al., 2016). It is not known to be antiviral in mammals. TRIM62 can restrict retroviruses in chickens, but no investigation of anti-IAV potential of this protein has been done in chickens or ducks.

avIFIT

Interferon-induced proteins with tetratricopeptide repeats (IFITs) are a family of proteins which have diverse functions in the cell such as mediating apoptosis, sequestering viral proteins and cell cycle regulation and have been extensively reviewed (Diamond and Farzan, 2013; Fensterl and Sen, 2015). IFITs have undergone duplication in mammals, fish and frogs, while ducks and chickens only have a single IFIT gene (avIFIT) (Zhou et al., 2013). Evolutionary analysis of duck avIFIT found that it most closely resembled mammalian IFIT5 (Wang et al., 2015; Rong et al., 2018a). Human IFIT5 is effective in restricting RNA virus replication by both interacting with immune signaling components (i.e., RIG-I and MAVS) (Zhang et al., 2013) and by binding 5′-ppp viral RNA (Abbas et al., 2013). In chickens, avIFIT (called IFIT5 by the authors) inhibits viral replication by interacting with 5′-triphosphate viral RNA and blocking subsequent replication steps (Santhakumar et al., 2018), similar to the mechanism of IFIT1 and IFIT5 in mammals (Abbas et al., 2013; Habjan et al., 2013).

Duck avIFIT is constitutively expressed in all tissues at basal levels but shows highest expression in digestive tissues such as intestine and stomach, although the expression levels in these tissues is still relatively low (Wang et al., 2015). To date we are unaware of any data on basal expression levels of avIFIT in the chicken. IFIT5 has low tissue specific expression in humans (Uhlen et al., 2015). Despite the slight differences in expression between humans and ducks, IFIT5/avIFIT is highly upregulated in both these species when induced by IFNs. Similarly, studies have demonstrated that avIFIT is upregulated during influenza infection in chicken intestinal epithelial cells when infected with LPAI (Kaiser et al., 2016) as well as in lungs of chickens infected with HPAI H5N1 (Ranaware et al., 2016).

When both human and chicken IFIT5 were overexpressed in chicken cells, they were found to inhibit viral replication and likewise, when chicken IFIT5 was knocked out from these cells, they were much more susceptible to infection (Santhakumar et al., 2018). Chicken avIFIT is found near the mitochondria in chicken cells, and as human IFIT5 interacts with both RIG-I and MAVS in infected cells, it would be worthwhile to investigate subcellular location of duck avIFIT. DF-1 cells were depleted of chicken avIFIT and transfected with duck avIFIT (Rong et al., 2018a). Duck avIFIT can inhibit IAV in DF-1 cells and was shown to do so by both upregulating IFNα/β and by binding the viral nucleoprotein (NP) from an H5N1 flu strain. This antiviral activity was not limited to only influenza virus, as in these experiments duck avIFIT also restricted double-stranded RNA and DNA viruses. Interestingly, in these DF-1 cells duck avIFIT also arrested cell growth in both infected and uninfected cells.

Mx

Mx1 is an ISG which is highly upregulated in response to viral infection, whose function and regulation as been recently reviewed (Haller et al., 2015). It acts as an antiviral effector and belongs to a large family of GTPases. Both humans and mice have two Mx genes while birds have one. Mx was found to be protective in laboratory mice, as many lab strains were found to have isoforms of Mx1 with exon deletions that left these mice more susceptible to influenza infection than mice with intact Mx1 (Lindenmann, 1962; Horisberger et al., 1983; Staeheli et al., 1988).

Mx is upregulated strongly in brain, lung and spleen of ducks that show a strong IFN response to infection (Smith et al., 2015; Saito et al., 2018). Mx alleles are highly variable in ducks (Dillon and Runstadler, 2010), however only a few of them have been experimentally analyzed for antiviral function. When transfected into mouse or chicken cells, duck Mx was not able to restrict IAV replication (Bazzigher et al., 1993). Chicken Mx weakly inhibits influenza, and that ability is dependent on the breed of chicken that the Mx was cloned from (Ko et al., 2002; Fulton et al., 2014), indicating high diversity in avian Mx. Chicken Mx also appears to be missing the GTPase activity of mammalian Mx proteins, suggesting this may be why antiviral activity has been weak at best in previous studies (Schusser et al., 2011). While more research on allelic variants and their potential to restrict IAV should be done, it is also possible that due to the close evolutionary relationship between IAV and ducks, the virus has evolved the ability to evade avian Mx during infection.

OASL

Interferon-inducible 2′-5′-oligoadenylate synthase (OAS) and OAS-like protein (OASL) are two related ISGs in humans, which are known to restrict influenza. OAS senses and degrades dsRNA through synthesis of oligoadenylates, which in turn switches on RNase L activity (Sarkar et al., 1999a,b; Justesen et al., 2000; Silverman and Weiss, 2014). RNase-L then degrades all mRNA in the cell (including ribosomal RNA), thus blocking viral replication. OASL inhibits viral replication independently of enzymatic activity by stabilizing the interaction of RIG-I and MAVS in a similar manner to that of ubiquitinylation by TRIM25. OASL has C-terminal ubiquitin-like domains that stabilize RIG-I CARD oligomers, thus potentiating downstream IFN signaling (Zhu et al., 2014; Ibsen et al., 2015). Birds do not appear to have OAS, but have OASL (Sokawa et al., 1984; Tag-El-Din-Hassan et al., 2018). Unlike human OASL, duck OASL has oligoadenylate synthetase activity, as well as the ability to restrict viral RNA in an RNase L independent manner (Rong et al., 2018b). It appears duck OASL functions as both human OAS and OASL, as it can activate both RNase L and RIG-I pathways. Chicken OASL has been found to inhibit WNV in mammalian cells (Tag-El-Din-Hassan et al., 2012). Chicken OASL is highly upregulated in tracheal epithelial cells 24 HPI (Jang et al., 2015). Both ostrich and duck OASL transfected into chicken DF-1 cells could control replication of both HPAI and LPAI influenza virus (Rong et al., 2018b). When OASL was knocked out of DF-1 cells, the cells became more permissive to influenza infection. Consistent with a role in augmenting innate signaling, overexpression of either ostrich or duck OASL also significantly increased the expression of RNase L, as well as other important immune effectors such as IFNα, IFNβ, IRF1, IRF7, Mx, and PKR.

PKR

The double-stranded RNA (dsRNA)-dependent protein kinase (PKR) is an ISG which functions as both an antiviral effector and anti-proliferative protein during infection (Garcia et al., 2006). PKR binds foreign dsRNA in the cytoplasm and autophosphorylates in order to become active, at which point it then phosphorylates eukaryotic initiation factor 2 (eIF-2α) causing broad inhibition of protein translation in the cell (Galabru and Hovanessian, 1987; Hovanessian, 1989). PKR has two N-terminal dsRNA-binding domains, which are both able to recognize viral RNA (Nanduri et al., 1998), and one C-terminal kinase domain.

PKR is an important antiviral effector in mice infected with IAV, as shown by the increased fatality rate of PKR knockout mice when infected with the H1N1 strain WSN (Balachandran et al., 2000). Chicken PKR has been functionally characterized and determined to be antiviral against VSV (Ko et al., 2004). Studies have shown that PKR is upregulated significantly during HPAIV H5N1 infection, even in lethal infections in the chicken where IFN production is limited (Daviet et al., 2009). The non-structural protein 1 (NS1) of IAV inhibits IFN responses in cells through interactions with OAS and PKR (Ma et al., 2010). Indeed, NS1 from HPAI H5N1 in a HPAI H7N9 background bound and inhibited PKR in chicken embryos.

PKR is upregulated in ducks infected with both HPAI and (to a lesser extent) LPAI virus (Fleming-Canepa et al., 2019) but to date we are unaware of any studies functionally characterizing duck PKR during influenza infection. We previously thought that ducks appeared to be missing the second dsRNA-binding domain (Fleming-Canepa et al., 2019), also confirmed by another group (Liu W. J. et al., 2018). However, through transcriptomic assembly done in our lab we have since found a transcript of the full-length PKR, which contains the second dsRNA-binding domain previously thought to be missing. This find suggests that ducks may predominantly express a splice variant of PKR missing the dsRNA-binding domain, or that this splice variant is preferentially amplified during PCR. Interestingly, it has been suggested that NS1 needs to bind both the kinase domain of PKR and residues 170–230 to keep PKR in an inactive conformation and prevent it from responding to dsRNA (Li et al., 2006). These residues correspond to the second RNA binding domain and the linker region of the protein. The two variants of duck PKR may allow ducks to respond to viral RNA despite NS1 antagonism. Duck PKR needs to be functionally characterized to determine not only its antiviral potential, but also expression levels of the full-length transcript.

Viperin

Viperin (RSAD2) is highly induced by Type I IFN, and many RNA virus infections. Viperin inhibits IAV by perturbing lipid rafts and thus inhibiting viral budding (Wang et al., 2007). Duck viperin is most highly expressed in blood, intestine, lung, and spleen in healthy birds (Zhong et al., 2015). Chicken viperin was upregulated in both spleen and lung of IAV

infected birds after 24 h (Goossens et al., 2015). It was also upregulated in chicken splenocytes as early as 6 h after poly (I:C) stimulation. In Newcastle disease (NDV) infected ducks, viperin was found to be highly upregulated after 24 h in the blood and peaked in expression in the lung and brain at 72 HPI (Zhong et al., 2015). Viperin is one of the most highly upregulated genes in duck lungs in response to H5N1 HPAI infection (Fleming-Canepa et al., 2019), however, the levels of viperin expression in chickens infected with the same strain of H5N1 was not mentioned (Smith et al., 2015). Ducks also significantly upregulated viperin in response to LPAI in the lung, but curiously not in the ileum (Fleming-Canepa et al., 2019).

IFITMs

Interferon-inducible transmembrane proteins (IFITMs) are upregulated upon viral infection, and have antiviral activity (Diamond and Farzan, 2013). This viral restriction usually happens during entry in either the early or late endosomes. Human IFITM1, IFITM2, and IFITM3 have all been shown to restrict IAV *in vitro* (Brass et al., 2009). The naming of the avian IFITMs has been complicated by the evolutionary history of gene duplication in this region during speciation, but sites for post-translational modifications identify IFITM3 as the gene next to B4GALNT4 (Smith et al., 2013), and the duck orthologs follow the same synteny (Blyth et al., 2016).

IFITM3 restricts IAV in both duck and chicken cells. Ducks upregulated all IFITMs including IFITM1, IFITM2, and IFITM3 in both lung and ileum during infection with HPAI, whereas chickens showed minimal upregulation of IFITMs (Smith et al., 2015). When duck IFITM1, IFITM2, IFITM3, and IFITM5 were overexpressed in DF-1 cells and challenged with LPAI, only IFITM3 significantly decreased viral infection (Blyth et al., 2016). Chicken IFITM3 is also able to restrict both IAV and lyssa virus in DF-1 cells (Smith et al., 2013). As IFITM1 and IFITM2 also control IAV in humans, it may be that host-pathogen co-evolution has allowed the virus to evade these proteins in ducks. Notably, duck IFITM1 has an insertion in exon 1, which changes the sub-cellular localization of the protein (Blyth et al., 2016), or it would restrict influenza. A 2017 study found that when duck IFITM2 was transfected into DF-1 cells it could control the replication of avian Tembusu virus (Chen et al., 2017). Avian Tembusu virus is a positive sense RNA virus belonging to the Flaviviridae family (Zhang et al., 2017). As IFITM2 restricts this virus but not IAV, it is possible that either the mammalian IFITM2 developed the ability to restrict IAV later in evolution, or that the avian strains we tested have evolved to escape from IFITM2. The upregulation of IFITM2 during IAV infection is most likely due to interferon stimulation and is not virus specific.

A NOTE ON MISSING GENES AND DARK DNA

Throughout this review we have spoken about genes that are presumed missing from ducks, chickens or birds in general. Because bird genomes contain many GC rich areas (Hron et al., 2015), they are notoriously hard to amplify using PCR based methods. As such, genes may be presumed missing in next generation sequencing applications, as well as with exploratory PCR based methods. This leaves many genes thought to not exist in birds, simply undiscovered. Such was the case with tumor necrosis factor alpha (TNFα), which for years was thought to not exist in chickens. It was recently cloned and characterized from chickens and found to have very low homology to mammalian orthologs, as well as have a high GC content (Rohde et al., 2018). We have also updated the full-length sequence of PKR that was formerly thought to be missing specific domains. One method to help with finding undiscovered genes from next gen sequencing data is using more advanced *de novo* assembly methods on combined RNAseq data. With advances in NGS technology and new software development to analyze fragmented GC rich RNAseq data, we will be able to better mine transcriptomes for genes as well as gain insight into avian immune system evolution. Furthermore, a wealth of genome information from many avian species is becoming available.

CONCLUSIONS

In this review, we summarize recent advances in understanding PRR in ducks, comparing them to chicken PRR, and analyzing their downstream signaling adaptors. We also investigated tissue expression of these innate immune components to try to gain insight into where these proteins were most expressed. Higher tissue expression of PRRs and their effectors may allow ducks to respond more quickly to IAV in a tissue-specific manner. A rapid and robust response that is quickly dampened could allow ducks to limit damage from inflammatory sequelae. Duck RIG-I and MDA5 are most highly expressed in the trachea, lung and intestines, areas of both HPAI and LPAI influenza replication (**Figure 2A**). However, the downstream adaptor molecules TBK1, TRAF3 and IRF7 are mostly expressed in digestive tissues with very little basal expression in the lung. This contrasts with chickens, which have high expression of these proteins in the lung. This pattern may circumvent out-of-control inflammatory reactions to HPAI and be protective to the duck, but further investigation is needed to confirm this. Duck TLR3 is most highly expressed in the trachea, while duck TLR7 is highest in the lung, fitting a similar pattern to the RLRs (**Figure 2B**). There appears to be a similar pattern of low lung expression of the adaptor of TLR3, TRIF, but there is no data on duck tracheal expression of TRIF to confirm this. Likewise, chicken TRIF basal expression has not yet been looked at in respiratory tissues. NLRP3 has high relative expression in chicken lungs, whereas ducks have higher basal expression in their hearts (**Figure 2C**). It is interesting to note that ducks seem to express PRRs at a high basal level in areas where influenza replicates, but the adaptor molecules are much less expressed in lung and respiratory tissues, the areas of HPAI replication. As more tissues are investigated, transcriptome mining for expression levels of these PRRs and adaptors, where missing, may help a complete picture to emerge.

Throughout this review we summarize the function and regulation of PRRs in chickens, ducks, and humans during IAV infection. While the differences in the RLR pathway are well-studied in ducks, there are currently few studies on TLR and NLR and their adaptor molecules in ducks during IAV infection. As these pathways converge and co-regulate each other, this is a very important piece of the story that is missing. Likewise, many proteins mentioned in this paper have been studied at the regulation level, but very few have been functionally and biochemically characterized.

We acknowledge that much of the work is yet to be done characterizing adaptor proteins in IFN and pro-inflammatory cytokine signaling networks. Investigation of these regulatory proteins in ducks and other birds, will allow us to see the conserved mechanisms, and find those that are not. Further, we acknowledge the bias that most immunological research looks at positive regulators of innate signaling. However, as ducks are equally adept at initiating and shutting down inflammatory responses, we should also begin to investigate inhibitory proteins and their expression and function. It should also be noted that functional studies in innate immunity in both ducks and chickens are limited. As such, data on PRR tissue expression and upregulation is often limited to small sample groups. Tissue expression can vary with age and breed of animals, and all studies discussed here used domestic breeds of ducks. When looking at tissue expression of genes as a potential route of resistance, it may be beneficial to also look at gene expression in wild mallards, which are constantly adapting and evolving with IAV. Indeed, it would be worthwhile understanding the allelic diversity of PRR genes and variation in function across many species of wild ducks. This may give us more insight into detection and resistance to IAV in its natural host and reservoir, the mallard duck.

AUTHOR CONTRIBUTIONS

LC originally drafted and edited the manuscript. KM edited the manuscript.

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Inferring the Urban Transmission Potential of Bat Influenza Viruses

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Bats are considered natural reservoirs of various, potentially zoonotic viruses, exemplified by the influenza A-like viruses H17N10 and H18N11 in asymptomatic Neotropical bats. These influenza viruses are evolutionarily distinct, are poorly adapted to laboratory mice and ferrets and cannot reassort *in vitro* with conventional strains to form new influenza subtypes. However, they have attracted renewed attention following reports that their entry in host cells is mediated by the trans-species conserved MHC-II proteins, suggesting that they hold zoonotic potential. Despite the recent studies, the viruses' epidemiology and public health significance remain incompletely understood. Delineating the mechanistic basis of the interactions with their hosts and assessing their global distribution are essential in order to fully assess the zoonotic threat that these strains pose.

Keywords: bats, influenza virus, haemagglutinin, neuraminidase, Major Histocompatibility Complex (MHC) class II, sialic acids

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INTRODUCTION

The Severe Acute Respiratory Syndrome (SARS), Middle East Respiratory Syndrome (MERS), Nipah (NiV), Hendra (HeV), and Ebola (EBOV) viruses' outbreaks confirmed the inextricable nature of human and bat health and disease and highlighted that focusing on the "spillover" potential of known, and novel, bat viruses is critical to predict and prevent pandemics. The remarkable ability of bats to coexist with a wide range of viruses that would be pathogenic in flightless mammals (Fl^{-}M) is not yet fully understood but possibly relates to their unique, flight-adapted antiviral immunity (Calisher et al., 2006; Hayman et al., 2013). Intriguingly, genetic material from viruses that resemble influenza type A viruses has been recovered from asymptomatic fruit bats of the Neotropic bat family *Phyllostomidae* (*Sturnira lilium* and *Artibeus planirostris*) in several countries of Central and South America (**Figure 1**) (Tong et al., 2012, 2013; Campos et al., 2019). Influenza A viruses (IAVs) are orthomyxoviruses with eight single-stranded negative-sense viral RNAs (vRNAs) encapsidated into viral ribonucleoproteins (vRNPs). IAVs emerge from aquatic birds, *via* genome reassortment and mutation, and are able to cause epidemics (and sporadic pandemics; Simonsen, 1999) in humans, lower animals and birds (Simonsen, 1999). The bat influenza viruses (BatIVs) are phylogenetically distinct from the conventional IAVs and they were designated as H17N10 and H18N11 (**Table 1**). Bats in Latin America, but not in Central Europe, have been found seropositive for BatIVs (Tong et al., 2013; Fereidouni et al., 2015). Antibodies against human H2N2 and H3N2, as well as classical H9, have also been found in bats elsewhere (L'vov et al., 1979; Kelkar et al., 1981; Isaeva et al., 1982), suggesting they are susceptible to IAV infection. The epithelial kidney cells of flying foxes (*Pteropus alecto*) co-express both avian (α2,3-Gal) and human (α2,6-Gal) sialic acid (SA) receptors (Chothe et al., 2017) and are thus susceptible to infection by both avian and human IAVs, but more importantly

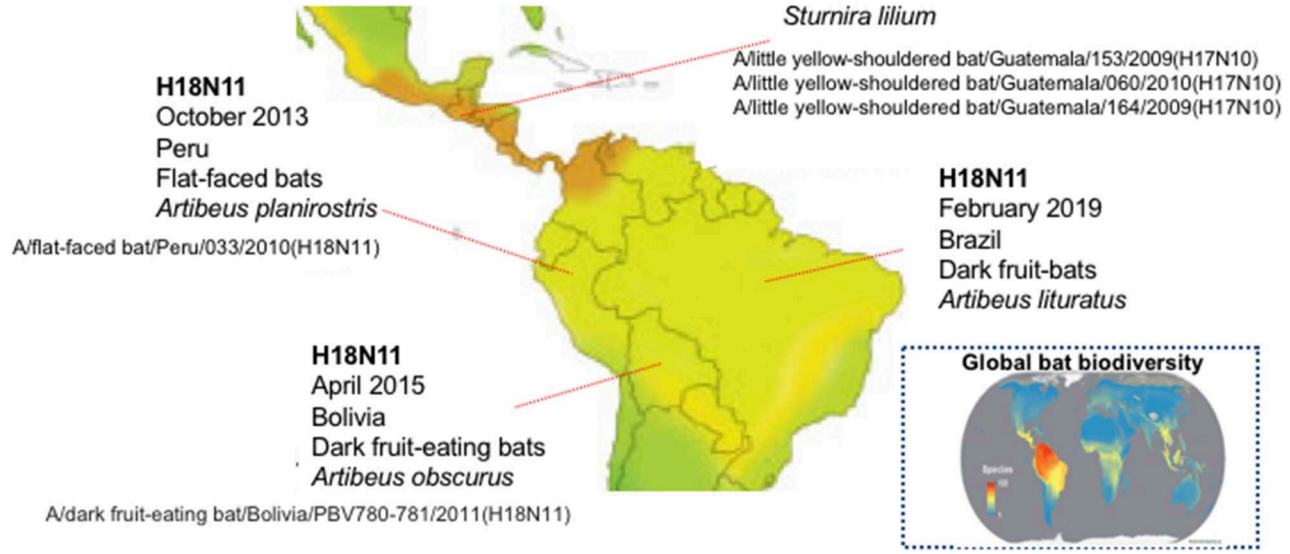

FIGURE 1 | Countries of Central and South America where bat influenza A viruses have been reported. Map showing the global pattern of bat species richness was provided by Clinton Jenkins (see BiodiversityMapping.org) using species data from The IUCN Red List of Threatened Species (2018; https://www.iucnredlist.org) (Jenkins et al., 2013).

they allow reassortment between co-infecting influenza viruses (Dlugolenski et al., 2013). The N-terminal domain of the H17N10 PA subunit of the influenza virus polymerase complex possesses endonuclease activity comparable to that of IAVs (Tefsen et al., 2014). Equally intriguingly, in the position 627 of the polymerase gene PB2, one of the most commonly identified IAV virulence markers, BatIVs have a serine compared to glutamic acid in avian and lysine in mammalian influenza strains, suggestive of an alternative evolutionary pathway for avian IAV's adaptation in mammals (Mehle, 2014). This all raised the question whether novel IAVs could emerge from bats to which human and animal populations would be immunologically "naïve," causing pandemics.

BatIVs ARE DISTINCT AND EMPLOY UNCONVENTIONAL RECEPTORS FOR CELL ENTRY

The genetic material of BatIVs is similar to classic flu viruses, but their surface glycoproteins haemagglutinin (HA) and neuraminidase (NA) are evolutionarily and functionally diverged (Li et al., 2012; Zhu et al., 2012; Sun et al., 2013; Tong et al., 2013). BatIVs cannot be cultured in embryonated chicken eggs and do not agglutinate red blood cells (Tong et al., 2012, 2013). Initial efforts by researchers to isolate live infectious BatIVs directly from bats failed, due to unavailability of permissive cell lines (Ciminski et al., 2017). In addition, research on these viruses was further complicated by the dearth of bat cell lines and the limited bat genomic data. Attempts to circumvent these limitations have included: (i) using HA-17 or HA-18 pseudotyped vesicular stomatitis virus (VSV) and HIV-1 based lentiviruses (Hoffmann et al., 2016; Maruyama et al., 2016; Carnell et al., 2018; Giotis et al., 2019), (ii) engineering BatIV/IAV chimaeric viruses (Juozapaitis et al., 2014; Zhou et al., 2014), and (iii) reconstructing authentic BatIVs using reverse genetics (Moreira et al., 2016; Sato et al., 2019; Zhong et al., 2019). HA17-VSV was able to infect bat cell lines (EidNi, HypNi, and EpoNi) but only a few of the common $Fl^{-}M$ cell lines, including human U-87 MG glioblastoma and SK-Mel-28 melanoma cells, canine RIE 1495 and MDCK II kidney cells (Hoffmann et al., 2016; Maruyama et al., 2016; Moreira et al., 2016). The identification of MDCK II, in particular, as susceptible cell lines to BatIVs opened the way for a more comprehensive characterization of these strains (Moreira et al., 2016; Giotis et al., 2019; Karakus et al., 2019).

Crystal structure analyses revealed that the bat haemagglutinins display typical HA protein folds but lack any obvious cavity to accommodate SA, which are the conventional receptors of IAVs. Recently, two independent studies demonstrated that the cell-entry of H17N10 (Giotis et al., 2019) and H18N11 (Karakus et al., 2019) is mediated by MHC-II receptors that are well-conserved in many species. Hence, immortalized cell lines that express MHC-II receptors on their surface such as several human leukemia and lymphoma cell lines Raji, Ramos, and BJAB B-lymphocytes could be used for the study of the viruses' biology (Giotis et al., 2019). Interestingly,

TABLE 1 | List of distinctive features of IAVs and BatIVs.

	IAVs	BatIVs
Known hosts	Birds, humans, swine, equine, and marine mammals	New World bats
Clinical manifestation	Mild to severe respiratory disease to humans and birds, cause outbreaks/epidemics, and sporadically pandemics	Asymptomatic (unclear)
Cell surface receptors/entry factors	Sialic acids	MHC-II
Role of haemagglutinin	Cell attachment/entry	Cell entry
Role of neuraminidase	Sialidase activity	Unknown
Culture in embryonated chicken eggs	Yes (usually)	No
Agglutination of red blood cells	Yes (usually)	No
Genetic drift	Yes	Yes
Genetic reassortment	Yes	Yes (not with IAVs)
Main transmission routes	Respiratory droplets, direct contact/fecal-oral	Fecal-oral (unclear)

ectopic expression of pig, mice, and chicken MHC-II have been shown to confer susceptibility to H18N11 in non-susceptible cells (Karakus et al., 2019) implying a potential role for the respective animals as intermediary hosts. It is as yet unclear whether MHC-II receptors function with other unknown factors to facilitate virus internalization and also whether the viruses remain cell associated following cell-entry and are being passed on by direct cell-cell contact.

MHC-II molecules occur as three highly polymorphic isotypes (HLA-DR, HLA-DP, and HLA-DQ). They are selectively expressed on the surface of professional antigen presenting cells (APCs), act as ligands for the T-cell receptor (TCR), and play a key role in the presentation of foreign antigens to $CD4^+$ T helper cells and immune surveillance (Jones et al., 2006; Roche and Furuta, 2015). Bats contain all classical MHC class II gene families that are responsible for antigen presentation with an extra *DRB2* gene copy located outside the MHC-II region in *P. alecto* (Ng et al., 2017). The MHC-II dependent cell entry suggests that BatIVs might hijack APCs such as B lymphocytes and dendritic cells for viral dissemination and/or survival perhaps in the early stages of infection. It is unknown to what degree their binding to APCs might influence the global outcome of the host immune responses. A blockade of TCR recognition by steric hindrance as described for Epstein-Barr virus (Ressing et al., 2003, 2005; Wiertz et al., 2007), could explain the asymptomatic status of the infection in the captured New World bats.

THE ENIGMATIC ROLE OF BAT INFLUENZA VIRUSES' NEURAMINIDASES

The bat neuraminidases (NAs) are structurally similar to classical NAs but lack conserved amino acids for SA binding or cleavage (Li et al., 2012; Zhu et al., 2012). Moreover, unlike classical NAs, they display no enzymatic activity (Garcia-Sastre, 2012; Carnell et al., 2018), have a dispensable role in viral entry (Hoffmann et al., 2016; Maruyama et al., 2016; Giotis et al., 2019) and their function is not yet elucidated. Recent studies demonstrated that the passage of reverse-genetics-generated H18N11 virus in cell cultures, accumulates mutations in the N11 protein that increase virus titers in culture and may enhance organ tropism *in vivo* (Zhong et al., 2019). Ciminski et al. demonstrated by utilizing a chimeric bat influenza virus (PR8-H18N11) that viruses encoding the full-length N11 protein exhibited a growth advantage over viruses that encode a truncated protein version and also showed that N11 is essential for viral transmission (Ciminski et al., 2019b). Another study showed that the N10 protein facilitates heterosubtypic (H5 and H7) influenza hemagglutinin-bearing pseudotype release in the absence of another source of neuraminidase, indicating a possible role of N10 in viral release (Carnell et al., 2018). It has been proposed that N11 downregulates MHC-II, thereby facilitating virion release but mechanistic data for such function is as yet missing (Ciminski et al., 2019b). There is no evidence that a functional balance exists between bat HAs and NAs although it is possible that the proteins coordinate their actions. Despite the recent progress in our understanding, the exact function of bat NAs remains an enticing mystery.

INTRASPECIES AND INTERSPECIES TRANSMISSION OF BAT INFLUENZA VIRUSES

It is now becoming more evident that BatIVs may transmit in a different manner than conventional flu strains. It has recently been shown that the H18N11 virus readily transmits between bats (Ciminski et al., 2019b). Following experimental intranasal H18N11 infection, the Neotropical *Phylostomidae* bat species *Artibeus jamaicensis* shed high viral loads via the fecal route and were able to infect naïve contact animals (Ciminski et al., 2019b). Histopathological analysis of the infected bats indicated that viral replication proceeds in the follicle-associated epithelium of gut-associated lymphoid tissue, suggesting virus uptake from the gastrointestinal lumen (Ciminski et al., 2019b), in line with the rich gut epithelial expression of MHC-II (Wosen et al., 2018). Furthermore, the researchers detected high loads of H18N11 viral transcripts in rectal swabs and excretions (Ciminski et al., 2019b). Collectively, these findings imply that an environmental (fecal-oral) mode of BatIVs transmission is more likely than an airborne one, albeit the latter is not yet compellingly disproved. Other infection routes including subcutaneous, transplacental, vaginal, intracranial infections have not yet been reported. In contrast to bats, H18N11 has been reported to have limited replicative ability in laboratory mice and more interestingly in ferrets which share similar lung physiology and SA distributions to humans (Ciminski et al., 2019b; Karakus et al., 2019; Zhong et al., 2019). Whether these observations are animal/infection-route-dependent or they actually reflect a low zoonotic risk for BatIVs remains to be seen.

In absence of conclusive scientific proof, the question remains as to whether BatIVs are confined to a sylvatic transmission cycle

and perpetuate in Neotropical bat populations or are capable of urban adaptation. Anthropogenic disruptions of ecological habitats that led to urban transmission of other enzootic bat viruses (i.e., HeV, NiV) have been extensively described in the scientific literature. Despite the increasing disturbances in the fire-prone Neotropical forests, New World bats have not yet been implicated in the transmission of zoonotic viruses, other than rabies, to humans (Moratelli and Calisher, 2015). Even so, Latin America is home to the richest and most diverse bat fauna in the world (**Figure 1**) including almost 150 *Phyllostomidae* species (Jenkins et al., 2013) in which BatIVs have been detected. Unlike African and Asian bats which are consumed regularly, New World bats are only eaten by few native indigenous people (Moratelli and Calisher, 2015). A possible exposure to infected bat blood and body fluids may hypothetically create a pathway for disease transmission to humans. Another scenario may involve the accidental introduction of BatIVs to the local fauna or other *Phyllostomidae* species such as the widespread blood-eating vampire bat (*Desmodus rotundus*), which thrives in both native and anthropogenically transformed ecosystems (Bergner et al., 2020). Vampire bats have long been suspected of passing on rabies to humans and livestock in Latin America by biting and scratching (Rupprecht et al., 2002). It will be useful to explore in future studies whether haematophagous bats can act as maintenance hosts for BatIVs and if their biting can form a potential zoonotic transmission route either directly or through mammalian intermediate hosts.

THE HOST FACTORS REGULATING BatIVs REPLICATION ARE POORLY UNDERSTOOD

BatIVs, like all viruses, have to compromise with positive and negative genetic factors present in target cells for their survival at each replication stage. Little is known regarding the interaction of BatIV proteins and RNA with the host or viral factors even though such interactions may determine the fate and/or efficiency of infection, transmission, and epidemic potential of the viruses. Previous studies revealed that the bat Nonstructural NS1 proteins can act as interferon (IFN) antagonists in human cells, and likely inhibit induction of IFN at a pretranscriptional level (Ciminski et al., 2017). More recently, it has been shown that the IFN-induced human MxA protein controls the replication of H18N11, but it is not clear whether sufficient MxA-escape mutations in H18N11 NP can be acquired *in vivo* that could potentially result in full MxA resistance (Ciminski et al., 2019a). Nearly all lab work examining host and viral immune-modulating proteins is performed with human/rodent cell lines. The difficulty in interpreting these data is that evolutionarily-optimized immune factors behave differently in non-natural hosts. Certainly, comprehensive kinetic analyses of immune-responses to BatIVs using primary or immortalized bat cell lines will be particularly informative. For instance, a comparison of the transcriptome of BatIV infected versus uninfected bat cells could help us identify specific immune genes contributing to host resistance and the molecular mechanisms underlying the viral pathogenesis.

DO BAT INFLUENZA VIRUSES POSE A ZOONOTIC RISK?

Reassortment of gene segments between co-infecting viruses is a key process mediating the genetic evolution of influenza viruses and the generation of novel epidemic and pandemic strains. BatIVs are able to reassort between themselves but not with conventional IAVs *in vitro* (Juozapaitis et al., 2014; Zhou et al., 2014). Generation of bat chimeric viruses was only possible when the HA/NA coding regions were flanked with the authentic BatIV packaging signals demonstrating packaging incompatibilities between IAVs and BatIVs (Juozapaitis et al., 2014; Zhou et al., 2014). This finding dismisses the scenario of emergence of a new "reassortant" virus with human/avian IAVs unless the bat viruses undergo major genetic changes over time.

However, the abilities of BatIVs to (i) reassort between themselves, (ii) to mutate in order to infect and transmit sustainably among their hosts, and (iii) enter human HLA-DR^+ cells, highlight that a zoonotic transmission of BatIVs is theoretically possible. The documented spillover of other non-reassortant bat-borne RNA viruses following continued host-pathogen interaction (i.e., NiV and EBOV) lends certain credence to this hypothesis, albeit clearly, supporting evidence is lacking.

To explore the ecological and evolutionary dynamics of these and possibly other unknown influenza-A-like viruses, further prevalence and serological studies in Neotropical bat populations are required coupled with the surveillance of bat-exposed humans and livestock. Surveys of bat colonies have previously led to identification of other zoonotic viruses, including HeV in Pteropus sp. in Australia, NiV in Pteropus lylei in Thailand, and MARV in Rousettus aegyptiacus in Uganda (Wacharapluesadee et al., 2010; Amman et al., 2012; Field et al., 2015). A computational study which used spatial empirical models to trace the steps of emergence of bat viruses and the transmission opportunities to humans pinpointed sub-Saharan Africa as the top-priority location for pathogen discovery in wildlife (Brierley et al., 2016). West Subsaharan Africa, in particular, hosts enormous populations of sedentary and migrating bats living in proximity to human and animal populations. Considering the number, the morbidity and mortality of emerging viruses that are hosted in African bats as well as the serological evidence against IAVs (Freidl et al., 2015), future surveillance and serological studies could lead to the identification of novel bat influenza subtypes.

CONCLUSIONS

In summary, BatIVs are unconventional influenza viruses that resemble to some extent more paramyxoviruses rather than typical orthomyxoviruses. Despite the recent findings on the cell entry factors and NAs of these viruses, it is clear that we only scratched the surface in terms of characterization of these viruses. The scientific evidence so far indicate a

limited spillover risk but data is not conclusive enough to dismiss out of hand the possibility of zoonotic transmission. Forecasting viral spillover is a challenging task and additional interdisciplinary and more up-to-date approaches are warranted to fully appreciate the ecology and the implications of these viruses for public health. Future studies on BatIVs hold extra value as they can provide broader mechanistic insights into the molecular biology of influenza viruses and might inform translational studies.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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The Stronger Downregulation of *in vitro* and *in vivo* Innate Antiviral Responses by a Very Virulent Strain of Infectious Bursal Disease Virus (IBDV), Compared to a Classical Strain, Is Mediated, in Part, by the VP4 Protein

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IBDV is economically important to the poultry industry. Very virulent (vv) strains cause higher mortality rates than other strains for reasons that remain poorly understood. In order to provide more information on IBDV disease outcome, groups of chickens ($n = 18$) were inoculated with the vv strain, UK661, or the classical strain, F52/70. Birds infected with UK661 had a lower survival rate (50%) compared to F52/70 (80%). There was no difference in peak viral replication in the bursa of Fabricius (BF), but the expression of chicken IFNα, IFNβ, MX1, and IL-8 was significantly lower in the BF of birds infected with UK661 compared to F52/70 ($p < 0.05$) as quantified by RTqPCR, and this trend was also observed in DT40 cells infected with UK661 or F52/70 ($p < 0.05$). The induction of expression of type I IFN in DF-1 cells stimulated with polyI:C (measured by an IFN-β luciferase reporter assay) was significantly reduced in cells expressing ectopic VP4 from UK661 ($p < 0.05$), but was higher in cells expressing ectopic VP4 from F52/70. Cells infected with a chimeric recombinant IBDV carrying the UK661-VP4 gene in the background of PBG98, an attenuated vaccine strain that induces high levels of innate responses (PBG98-VP4^{UK661}) also showed a reduced level of IFNα and IL-8 compared to cells infected with a chimeric virus carrying the F52/70-VP4 gene (PBG98-VP4$^{F52/70}$) ($p < 0.01$), and birds infected with PBG98-VP4^{UK661} also had a reduced expression of IFNα in the BF compared to birds infected with PBG98-VP4$^{F52/70}$ ($p < 0.05$). Taken together, these data demonstrate that UK661 induced the expression of lower levels of anti-viral type I IFN and proinflammatory genes than the classical strain *in vitro* and *in vivo* and this was, in part, due to strain-dependent differences in the VP4 protein.

Keywords: IBDV, virulence, type I IFN, inflammation, cytokines, VP4

INTRODUCTION

Infectious bursal disease virus (IBDV) is a highly contagious, immunosuppressive virus belonging to the *Birnaviridae* family (Hoerr, 2010). The virus is non-enveloped, with a bi-segmented double stranded (ds) RNA genome encoding 3 open reading frames (ORFs) which are translated and processed to produce 5 viral proteins (VP1-5). Ranking among the top five infectious problems of chickens (Cazaban et al., 2017), IBDV poses a continuous threat to the poultry industry though economic losses and welfare concerns. Moreover, as the virus has a preferred tropism for B cells, the majority of which reside in the bursa of Fabricius (BF), surviving birds are often immunosuppressed, less responsive to vaccination programmes, and more susceptible to secondary infections (Giambrone, 1979; Spackman et al., 2018).

Disease severity depends on numerous factors including the age and breed of the bird, and the virulence of the infecting IBDV strain (Mahgoub et al., 2012). Since the first identification of IBDV in the 1960s, classical (c) strains have circulated worldwide, however, in the 1980s, so-called "very virulent" (vv) strains emerged, complicating IBDV control efforts (Brown et al., 1994; Brown and Skinner, 1996). The vvIBDV strains cause a far higher mortality rate than classical strains, reaching up to 60–70% in some flocks (van den Berg et al., 2000). However, the molecular basis for the difference in disease outcome remains poorly understood, although it has been demonstrated that both segments A and B contribute to virulence (Escaffre et al., 2013). Segment A encodes the non-structural protein, VP5, and a polyprotein (VP2-VP4-VP3) which is co-translationally cleaved by the protease, VP4 (Lejal et al., 2000). VP2 is the capsid protein and VP3 is a multifunctional scaffolding protein that binds the genome. The single ORF on Segment B encodes VP1, the RNA-dependent RNA polymerase.

The innate immune response to IBDV infection is characterized by the production of type I IFN responses, including the upregulation of IFNα and IFNβ, that lead to the induction of interferon stimulated genes (ISGs), including MX1, which is one of the top ISGs identified in chicken cells ranked by fold change (Giotis et al., 2017). The IFN response aims to provide an antiviral state in infected and bystander cells. In addition, pro-inflammatory cytokines, for example IL-6, IL-8, and IL-1β are produced following IBDV infection that recruit immune cells into the infected BF (Guo et al., 2012; Carballeda et al., 2014; Quan et al., 2017; He et al., 2018).

We sought to identify IBDV virulence determinants in order to better understand the molecular basis of phenotypic differences between vv- and c-IBDV strains. Here we report that vvIBDV UK661 down-regulated the expression of antiviral type I IFN responses and pro-inflammatory cytokines compared to cIBDV F52/70 *in vitro* and *in vivo*. Moreover, we demonstrate that the differences in IFN antagonism were, in part, due to strain-dependent differences in the VP4 proteins.

MATERIALS AND METHODS

Cells and Viruses

DF-1 cells (chicken embryonic fibroblast cells, ATCC number CRL-12203) were sustained in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, Merck, UK) supplemented with 10% heat inactivated fetal bovine serum (hiFBS) (Gibco, Thermo Fisher Scientific, UK) (Complete DMEM). DT40 cells (immortalized chicken B cell line, Baba et al., 1985) were maintained in Roswell Park Memorial Institute (RPMI) media supplemented with L-glutamine (Sigma-Aldrich, Merck), sodium bicarbonate (Sigma-Aldrich, Merck) 10% hiFBS, tryptose phosphate broth (Sigma-Aldrich, Merck) sodium pyruvate (Gibco), and 50 mM beta-mercaptoethanol (B-ME) (Gibco) (complete RPMI media). A vv-strain, UK661 (Brown and Skinner, 1996), and a c-strain, F52/70 (Bayliss et al., 1990) of IBDV were kind gifts from Dr. Nicolas Eterradossi, ANSES, France. Stocks of both viruses were generated by inoculating 3 week old specific pathogen free (SPF) Rhode Island Red (RIR) chickens and harvesting the BF at 3 days post-infection. BF tissue from 18 birds was pooled, homogenized, and the homogenate mixed with Vertrel XF (Sigma-Aldrich, Merck) and centrifuged at 1,200 g for 30 min. The resulting aqueous phase was harvested and frozen at −80°C. The cell-culture adapted vaccine strain, PBG98, and chimeric viruses within the PBG98 backbone (PBG98-VP4^{UK661} and PBG98-VP4$^{F52/70}$) were propagated in DF-1 cells. Briefly, flasks of DF-1 cells were inoculated with viruses and incubated until cytopathic effect (cpe) was observed, whereupon the supernatant was harvested, centrifuged at 1,200 rpm to pellet debris, and the supernatant was aliquoted and frozen at −80°C.

Virus Titration by $TCID_{50}$

In order to titrate the UK661 and F52/70 viruses, 96-well U-bottomed plates (Thermo Fisher Scientific) were seeded with the immortal B cell line, DT40, at a seeding density of 1×10^4 cells per well in 180 μL media. A 10-fold dilution series of the UK661 or F52/70 viruses was added to the cells, with 20 μL of each dilution added to each well in quadruplicate. Cells were incubated at 37°C for 5 days, fixed in 4% paraformaldehyde and stained with a primary mouse monoclonal antibody raised against IBDV VP2 (Wark, 2000) and a secondary goat anti-mouse antibody conjugated to Alexa Fluor 488 (Thermo Fisher Scientific). Wells were marked positive or negative for the presence or absence of virus by immunofluorescence microscopy and the $TCID_{50}$/mL calculated by the Reed and Muench method (Reed and Muench, 1938). In order to titrate the PBG98 and chimeric viruses, 96-well plates were seeded with DF-1 cells at a density of 1×10^4 cells per well in 180 μL media. A 10-fold dilution series of the PBG98 or chimeric viruses were added to the cells, with 20 μL of each dilution added to each well in quadruplicate. Cells were incubated at 37°C for 5 days, and wells were marked positive or negative for the presence or absence of cpe and the $TCID_{50}$/mL calculated by the Reed and Muench method (Reed and Muench, 1938).

F52/70 and UK661 *In vivo* Study

Forty-two SPF RIR chickens of mixed gender were obtained from the National Avian Research Facility (NARF) and reared at The Pirbright Institute. Chickens were randomly designated into mock-infected ($n = 6$), F52/70-infected ($n = 18$) and UK661-infected ($n = 18$) groups. At 3 weeks of age, birds were inoculated with either PBS or a virus dose of 1.8×10^3 $TCID_{50}$/bird, delivered intranasally, 50 μL per nares. Clinical scores were

recorded at least twice daily according to a points-based scoring system (**Supplementary Figure 1**) that characterized disease as mild (1–7), moderate (8–11), or severe (12–17). The scoring system was developed at The Pirbright Institute and approved by the Home Office (Project License number 7008981). Briefly, birds were scored on their appearance, behavior with and without provocation and handling, and included an assessment of the wattles, combs, feathers, eyes, posture, breathing, interactions with the rest of the flock, ability to evade capture, weight, and crop palpation. Six birds from each infected group were humanely culled by cervical dislocation at the time points indicated, or when the humane end point (a score of 11) was reached, and tissues were harvested at post-mortem for downstream analysis. The BF was harvested from each bird and divided into two sections, one stored in RNAlater (Thermo Fisher Scientific) for RNA extraction and one snap frozen on dry ice for virus titration by $TCID_{50}$. All animal procedures conformed to the United Kingdom Animal (Scientific Procedures) Act (ASPA) 1986 under Home Office Establishment, Personal and Project licenses, following approval of the internal Animal Welfare and Ethic Review Board (AWERB) at The Pirbright Institute.

PBG98, PBG98-VP4$^{F52/70}$, and PBG98-VP4^{UK661} *In vivo* Study

Seventy-two SPF RIR chickens of mixed gender were obtained from the NARF and reared at The Pirbright Institute. Chickens were randomly divided into mock-infected ($n = 18$), recombinant wild-type (wt) PBG98-infected ($n = 18$), PBG98-VP4$^{F52/70}$-infected ($n = 18$), and PBG98-VP4^{UK661}-infected ($n = 18$) groups. At 3 weeks of age, birds were inoculated with either PBS or a virus dose of 1.8×10^3 $TCID_{50}$/bird, delivered intranasally, 50 μL per nares. Clinical scores were recorded at least twice daily according to the points-based scoring system (**Supplementary Figure 1**). Six birds from each infected group were humanely culled at 2, 4, and 14 days post-infection and the BF was harvested from each bird and divided into two sections, one stored in RNAlater (Thermo Fisher Scientific) for RNA extraction and one snap frozen on dry ice. All animal procedures conformed to the United Kingdom ASPA 1986 under Home Office Establishment, Personal and Project licenses, following approval of the internal AWERB at The Pirbright Institute.

RNA Extraction, RT, and qPCR

RNA was extracted from 30 mg of homogenized bursal tissue, or from cells in tissue culture wells, using the Monarch Total RNA Miniprep Kit (NEB) according to the manufacturer's instructions. Complementary DNA (cDNA) was generated using SuperScript III Reverse Transcriptase (Invitrogen). The reaction constituents and conditions were consistent with the manufacturer's instructions. For virus quantification, qPCR was performed using TaqMan™ Universal qPCR Master Mix (Applied Biosystems) according to manufacturer's instructions. Amplification and detection of targeted genes was performed with the QuantStudio™ 5 (Applied Biosystems) with the following cycling conditions: 50°C for 5 min, 95°C for 2 min, 40°C cycles of 95°C for 3 s, and 60°C for 30 s. For quantification of host genes, a SYBR green qPCR was performed using Luna® Universal qPCR mix (NEB) according to manufacturer's instructions. Amplification and detection of targeted genes was performed with the QuantStudio™ 5 (Applied Biosystems) with the following cycling conditions: 95°C for 20 s, 40 cycles of 95°C for 1 s, and 60°C for 20 s, then a melt curve step at 95°C for 1 s, 60°C for 20 s, and 95°C for 1 s. Primers for all qPCR reactions can be found in **Supplementary Table 1**. CT values were first normalized to a housekeeping gene (RPLPO) and then to mock inoculated controls and expressed as fold change in a ΔΔCT analysis.

In vitro IBDV Infection

DF-1 cells were seeded into 24-well plates at 1.5×10^5/well and incubated at 37°C overnight to allow adhesion. Virus stocks were diluted in complete DMEM media to the specified multiplicity of infection (MOI) and added to the cells. DT40 cell suspensions were counted, pelleted and resuspended in a solution of virus diluted in complete RPMI media at the specified MOI. Cells were incubated for 1 h at 37°C and 5% CO_2. After incubation, the inoculum was removed from the cells and cells were washed with fresh media before incubation in fresh media at 37°C and 5% CO_2 until the desired time point.

Luciferase Assays for IFNβ

VP4 expression plasmids were designed and synthesized with the gene encoding a chicken codon-optimized enhanced (e) GFP tag at the 5′ end of the VP4 nucleotide sequence. DF-1 cells were seeded into a 24-well plate at a density of 1.5×10^5 cells/ well and incubated for 24 h at 37°C and 5% CO_2 until 80% confluency. Cells were transfected with expression plasmids using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. A final concentration of 500 ng plasmid per well was used following optimization experiments. Briefly, cells were co-transfected with 40 ng Renilla luciferase, 80 ng of a pGL3 Luciferase reporter plasmid containing the promoter regions of IFNβ upstream of a Firefly luciferase gene to measure type I IFN induction (a kind gift from Steve Goodbourn of St. George's, University of London) and 500 ng of eGFP or eGFP-tagged VP4 expression plasmids. Twenty four hours post-transfection, transfection efficiency was confirmed by immunofluorescence microscopy, and protein was extracted from parallel wells for western blot analysis. IFNβ production was stimulated by transfecting cells with (10 μg/mL) polyinosinic: polycytidylic acid (poly I:C) (Sigma Aldrich, Merck). Cells were lysed 6 hours after poly I:C transfection using 100 μL 1X passive lysis buffer (Promega). Plates were frozen for at least 30 min at −80°C before thawing and reading on a GloMax Multi plate reader (Promega). Briefly, once thawed, 10 μL of the sample was added in triplicate to a 96 well opaque white plate (Pierce) and analyzed on the plate reader using Stop and Glo reagents (Promega) according to the manufacturer's instructions. Firefly and Renilla values were recorded and Firefly luciferase values were normalized to Renilla values.

Western Blot

Cells were lysed in Laemmli Sample buffer (BIO-RAD) containing B-ME, heated to 95°C for 5 min, and disrupted by sonication. Samples were subject to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a Mini-PROTEAN tetra vertical electrophoresis chamber (BIO-RAD) and transferred to a nitrocellulose membrane using a Trans-Blot Turbo Transfer System (BIO-RAD). Membranes were stained with rabbit anti-VP4 [a gift from Jóse Castón of Centro Nacional de Biotecnología (CNB-CSIC)] and mouse anti-βactin (Thermo Fisher Scientific), followed by donkey anti-rabbit-680 and donkey anti-mouse-800 (LI-COR) and imaged with an Odyssey CLx (LI-COR).

Generation of Chimeric VP4 Viruses

A reverse genetics system for the cell-adapted (ca-)IBDV vaccine strain PBG98 was developed in house (Campbell et al., 2020). Briefly, the sequences of PBG98 segments A and B (Genbank Accession Numbers: MT010364 and MT010365) including the 5′ and 3′ non-coding regions were flanked by self-cleaving ribozymes (a hammerhead ribozyme upstream and a hepatitis delta ribozyme downstream, Chowrira et al., 1994). The whole sequence was ordered (GeneArt, ThermoFisher Scientific, UK) and cloned into a pSF-CAG-KAN vector (Sigma-Aldrich, Merck, UK) using restriction enzyme pairs Kpn1/ Nhe1 (Segment A) and Sac1/Xho1 (Segment B) (New England Biosciences, UK) to make two "reverse genetics" plasmids (pRGs), pRG-PBG98-A, and pRG-PBG98-B. DF-1 cells at 70% confluency were transfected with both plasmids with lipofectamine 2000 (Invitrogen, ThermoFisher scientific, UK) in order to rescue the recombinant PBG98 virus. Genestrings were synthesized for the PBG98 segment A, replacing the VP4 sequence with that of UK661 or F52/70 (PBG98-VP4^{UK661} and PBG98-VP4$^{F52/70}$, respectively) (GeneArt, Thermo Fisher). Genestrings were digested using KpnI and NheI and ligated into the pSF-CAG-KAN vector to make the "reverse genetics" plasmids pRG-PBG98-VP4^{UK661} or pRG-PBG98-VP4$^{F52/70}$. These plasmids were transfected into DF-1 cells with pRG-PBG98-B in order to rescue the PBG98-VP4^{UK661} and PBG98-VP4$^{F52/70}$ viruses. Transfected DF-1 cells were incubated until cytopathic effect (cpe) was observed, after which the virus-containing supernatants were passaged onto additional DF-1 cells to generate viral stocks. The sequence of the VP4 gene was determined by Sanger sequencing all recovered recombinant viruses.

Statistical Analysis

Statistical significance between experimental groups was determined following a Shapiro-Wilk normality test to confirm whether the data followed a normal distribution for parametric or non-parametric testing, and a one-way ANOVA with Tukey's multiple comparison test on fold change values was performed, using Minitab (v.18). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

RESULTS

UK661 Was More Virulent Than F52/70 but Both Replicated to the Same *In vivo* Peak Titer

Groups of 18 chickens were either inoculated with UK661 or F52/70, and 6 chickens were mock-inoculated with PBS alone. Birds were assessed clinically at least twice daily, and humanely culled when humane end-points were reached. At 24 and 48 h post-infection (hpi), 6 birds per infected group were humanely culled and the BF harvested for quantification of viral replication and host gene expression. At 54 hpi, 3 of the remaining 6 (50%) birds inoculated with UK661 reached their humane end-points and were humanely culled, compared to 1/6 (17%) of birds inoculated with F52/70 (**Figure 1A**), consistent with UK661 being more virulent than F52/70, as expected. The remaining infected and mock-inoculated birds were humanely culled at 72 hpi. There was no statistically significant difference in the BF: body weight ratio (BF:BW) between groups of birds (**Supplementary Figure 2**), a metric that is sometimes used as a surrogate of bursal pathology (Mase and Oishi, 1986; Cloud et al., 1992). Moreover, the kinetics of disease progression was similar between the two viral strains, peaking at 54 hpi (**Figure 1B**), and there was no significant difference in the fold change in viral transcripts measured by RTqPCR between the two strains at any of the time points measured (**Figure 1C**). We also determined the viral titers in the bursal tissue at each time point by $TCID_{50}$, and although we found UK661 replicated to a lower titer than F52/70 at 24 hpi ($^{*}P < 0.05$), there was no significant difference in viral replication at later time points, which peaked at ∼8 $\log_{10}$ $TCID_{50}$/g of bursal tissue for both strains (**Figure 1D**).

The Expression of Type I IFN and Pro-inflammatory Genes Was Significantly Reduced in BF Tissue Harvested From Birds Infected With Strain UK661 Compared to Strain F52/70 *In vivo*

RNA was extracted from BF samples and reverse transcribed to cDNA that was used as the template in qPCR assays targeting chicken type I IFN genes IFNα, IFNβ and Mx1, and pro-inflammatory cytokines IL-6, IL-8, and IL-1β. These genes were selected as they are the most relevant to studying antiviral type I IFN and pro-inflammatory responses, and have previously been shown to be upregulated following IBDV infection (Guo et al., 2012; Carballeda et al., 2014; Giotis et al., 2017; Quan et al., 2017; He et al., 2018). The mean expression of IFNα (**Figure 2A**), IFNβ (**Figure 2B**), and Mx1 (**Figure 2C**) was lower in BF samples harvested from UK661-infected birds compared to F52/70-infected birds at 24 and 48 h post-inoculation, which was statistically significant for IFNβ at 48 hpi and Mx1 at 24, 48, and 72 hpi ($^{*}P < 0.05$). There was no significant difference in the mean expression of IL-1β (**Figure 2D**) or IL-6 (**Figure 2E**) between birds infected with the two strains, but IL-8 was significantly reduced in BF samples

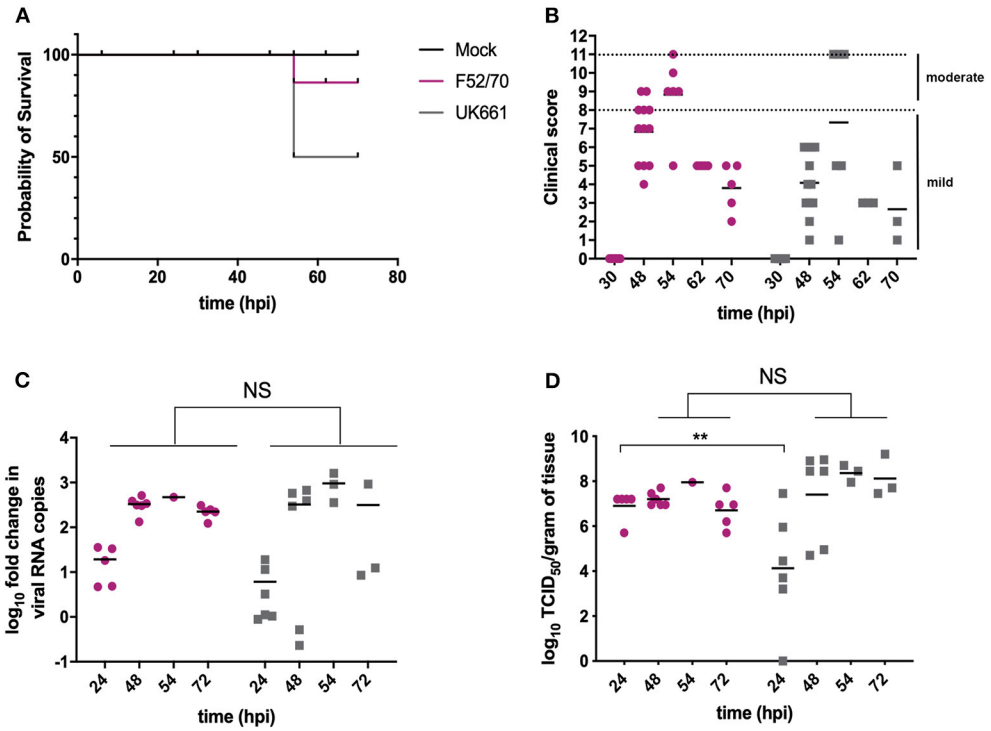

FIGURE 1 | The UK661 strain was more virulent than the F52/70 strain, but both strains replicated to the same peak titer *in vivo*. Birds were checked twice daily by two independent observers for clinical signs and a Kaplan Meier survival curve plotted of mock- (black), F52/70- (pink), and UK661- (gray) inoculated birds that reached their humane end points (clinical score of 11) **(A)**. Clinical signs were quantified by a scoring system and divided into mild (1-7) and moderate (8-11). Each bird was assigned a clinical score at the indicated time points post-infection **(B)**. Six birds per group were humanely culled at 24 and 48 h post-infection (hpi), one F52/70 and three UK661-infected birds reached their humane end-points at 54 hpi and the remaining birds were culled at 72 hpi. The bursa of Fabricius was harvested at necropsy and the $\log_{10}$ fold change in viral RNA copies/g tissue determined by RT-qPCR **(C)**. The infectious titer was determined by titration onto DT40 cells in the method described by Reed and Muench. Virus titers were expressed as $\log_{10}$ $TCID_{50}$/g of tissue **(D)**. The horizontal lines are the mean values. Data passed a Shapiro-Wilk normality test before being analyzed by a one-way ANOVA and a Tukey's multiple comparison test (**$p < 0.01$).

harvested from UK661-infected birds compared to F52/70-infected birds at 48 and 72 h post-inoculation (*$P < 0.05$) (**Figure 2F**). Taken together, these data demonstrate that the expression of antiviral type I IFN responses (IFNβ and Mx1) and pro-inflammatory cytokine responses (IL-8) in the BF was reduced following infection with the vv strain compared to the classical strain.

The Expression of Type I IFN and Pro-inflammatory Genes Was Significantly Reduced in B Cells Infected With UK661 Compared to F52/70 *In vitro*

We compared the replication of UK661 and F52/70 and the expression of type I IFN and proinflammatory cytokines in DT40 cells, an immortalized avian B cell-line. Cells were infected with UK661 or F52/70 (MOI 0.1) and the expression of virus and host-cell transcripts quantified by RTqPCR at 14, 48, and 72 hpi. UK661 replicated to a significantly higher titer than F52/70 across all time points (***$P < 0.001$) (**Figure 3A**). However, cells infected with UK661 showed significantly reduced expression of IFNα, IFNβ, and Mx1 compared to cells infected with F52/70 at 14, 48, and 72 hpi (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) (**Figures 3B–D**). Expression of IL-1β and IL-6 was also significantly reduced in cells infected with UK661 compared to F52/70 at multiple time-points (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) (**Figures 3E,F**). In contrast, IL-8 expression was significantly higher in cells infected with UK661 than F52/70 at 48 hpi (***$P < 0.01$) (**Figure 3G**). Taken together, these data suggest that the vv strain is able to reduce the expression of mRNAs for IFNα, IFNβ, Mx1, and the pro-inflammatory cytokines IL-1β and IL-6 to a greater extent than the classical strain *in vitro,* confirming our *in vivo* data.

The UK661-, but Not F52/70-, VP4 Protein Antagonized IFNβ Induction *In vitro*

The VP4 protein of IBDV has previously been identified as an IFN antagonist (Li et al., 2013). We hypothesized that strain-dependent differences in the VP4 proteins would be responsible for the observed differences in type I IFN responses between the strains. In order to address this, VP4 sequences across groups of very virulent, classical, and attenuated/cell-adapted IBDV strains available on the NCBI database were aligned using Clustal Omega (Sievers et al., 2011) (**Supplementary Figure 3A**). Compared to F52/70 VP4, there were 9 amino acids different in the UK661 VP4

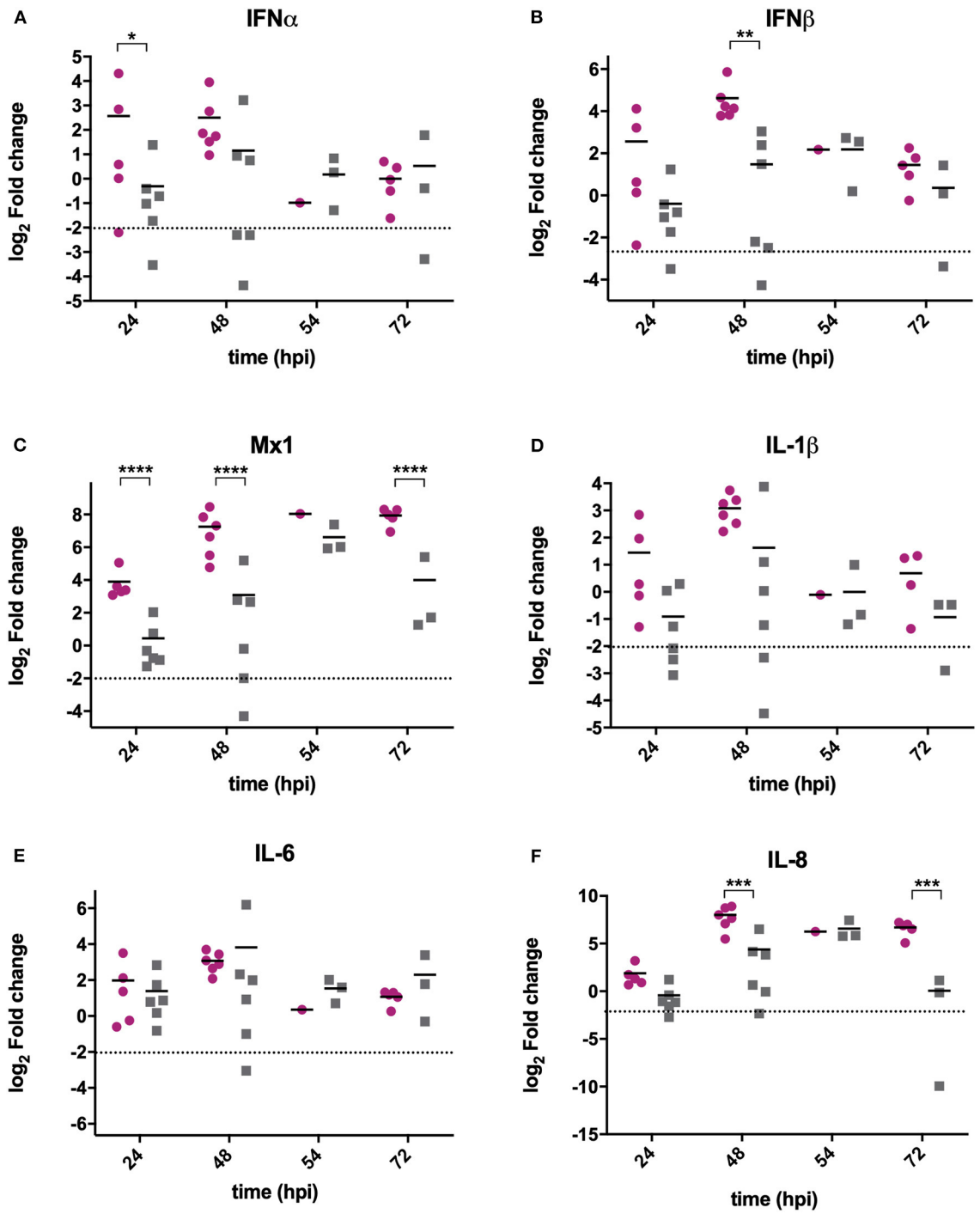

FIGURE 2 | The expression of type I IFN and pro-inflammatory genes was significantly reduced in BF tissue harvested from birds infected with strain UK661 compared to strain F52/70 *in vivo*. The bursa of Fabricius was harvested from mock and infected birds at necropsy and RNA extracted. RNA was reverse transcribed and amplified by quantitative PCR using specific primer sets for target genes. IFNα **(A)**, IFNβ **(B)**, Mx1 **(C)**, IL-1β **(D)**, IL-6 **(E)**, and IL8 **(F)**. The CT values were normalized to the housekeeping gene RPLPO and the $\log_2$ fold change in gene expression determined for the infected samples relative to the mock-infected samples in a ΔΔCT analysis and plotted for individual birds. The horizontal lines are the mean values. Data are representative of at least three replicate experiments and passed a Shapiro-Wilk normality test before being analyzed by a one-way ANOVA and a Tukey's multiple comparison test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). The dashed horizontal line represents the cut-off, below which genes were significantly down-regulated compared to mock-inoculated birds.

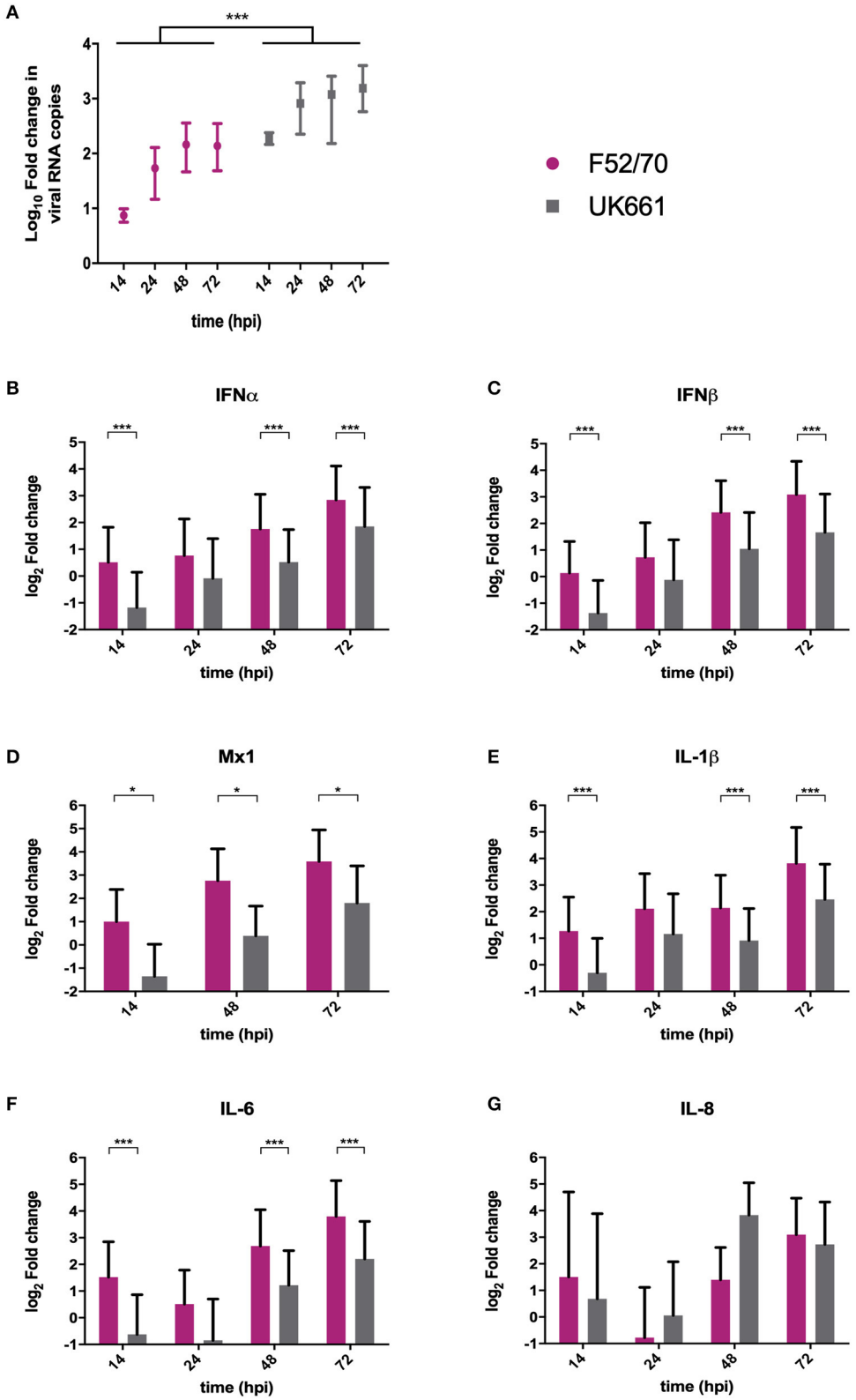

FIGURE 3 | The expression of type I IFN and pro-inflammatory genes was significantly reduced in B cells infected with strain UK661 compared to strain F52/70 *in vitro*. DT40 Cells were infected at an MOI of 0.1 with either the UK661 or F52/70 IBDV strains, or mock-infected with media alone and RNA was extracted from the

(Continued)

FIGURE 3 | cells at the indicated time points post-infection. RNA was reverse transcribed and amplified by qPCR using specific primer sets. The CT values were normalized to the housekeeping gene RPLPO and the $\log_{10}$ fold change in virus gene expression determined for the infected samples relative to the mock-infected samples in a ΔΔCT analysis and plotted **(A)**. The $\log_2$ fold change in host-cell gene expression was also determined for the infected samples relative to the mock-infected samples in a ΔΔCT analysis and plotted **(B–G)**. Data subsequently passed a Shapiro-Wilk normality test before being analyzed by a one-way ANOVA and a Tukey's multiple comparison test (*$P < 0.05$) **(A)**, or a two-tailed unpaired Student's *t*-test (*$p < 0.05$, ***$p < 0.001$). Data shown are representative of at least three replicate experiments, columns represent the mean values, and error bars represent the standard deviation of the mean.

protein (V31I, D114V, D122G, R132K, N141S, C170Y, K175N, P205S, and H241D). Interestingly, 5 of these were also found in other vv strains, from diverse geographical regions, but not in classical or vaccine strains (31I, 170Y, 175N, 205S, and 241D). The VP4 protein structures were modeled (using PyMOL version 2.0, Schrödinger, LLC) to the known VP4 structure of another member of the *Birnaviridae* family, the Yellowtail Ascites Virus (YAV; 4izk.2.A) (**Supplementary Figure 3B**), as the structure of IBDV VP4 protein remains unsolved at the time of writing. The YAV VP4 sequence shares 24.75% amino acid identity and lacks the last 25 amino acids of the IBDV sequence so the amino acid at position 241 is absent. Nevertheless, based on this model, the amino acid differences between the two VP4 proteins were found to cause some alterations in the predicted secondary structure, for example the presence of β-strands in the UK661 VP4 molecule that were not seen in the F52/70 VP4 molecule (**Supplementary Figure 3B**, dashed boxed regions).

To determine whether the VP4 proteins from UK661 and F52/70 IBDV inhibited IFNβ induction, we co-transfected DF-1 cells with an IFNβ luciferase reporter plasmid and plasmids encoding VP4 proteins from either UK661 or F52/70, tagged with enhanced GFP (eGFP) at the N-terminus (eGFP-UK661-VP4 and eGFP-F52/70-VP4). The eGFP tag was used to monitor transfection efficiency. Transfected cells were stimulated with poly I:C to induce the production of IFNβ that was quantified by measuring Firefly luciferase units normalized to Renilla luciferase. A low level of IFNβ induction was observed in all groups in the absence of poly I:C stimulation, with little difference between those transfected with the VP4 expression plasmids and a plasmid expressing eGFP alone (vector control plasmid) (**Figure 4**). In contrast, upon stimulation with poly I:C, there was an increase in IFNβ induction in cells expressing eGFP alone that was significantly reduced in cells expressing eGFP-UK661-VP4 (**$P < 0.01$). This reduction was not seen in cells expressing eGFP-F52/70-VP4, despite more protein detected by western blot than eGFP-UK661-VP4. In fact, there was a significant increase in IFNβ induction compared to cells expressing either eGFP-UK661-VP4 or eGFP alone (**$P < 0.01$). These data demonstrate that the VP4 protein from the vv IBDV strain down-regulates IFNβ induction *in vitro*, but the VP4 protein from the classical strain does not.

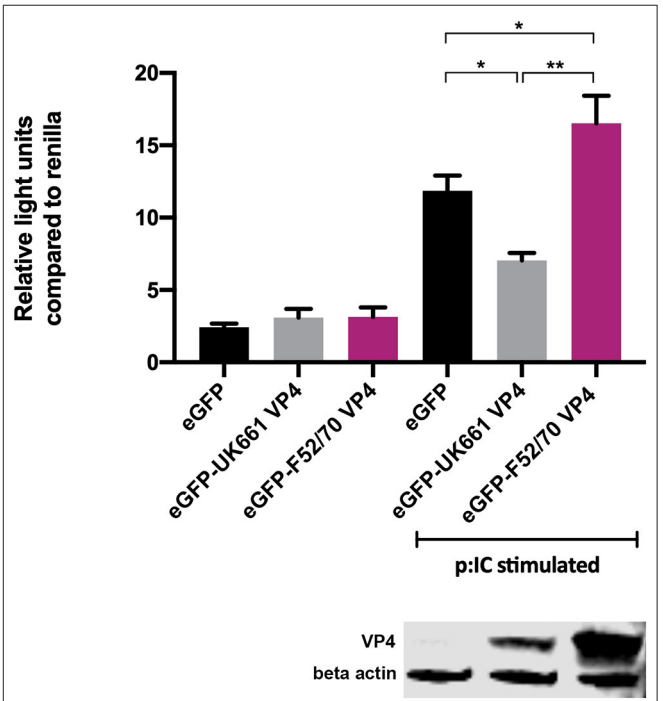

FIGURE 4 | The VP4 protein from the UK661 strain antagonized IFNβ induction, but the VP4 protein from the F52/70 strain did not *in vitro*. DF-1s were transfected with the chicken IFNβ promoter Firefly luciferase reporter and a constitutively active Renilla expression plasmid and 500 ng of either eGFP-UK661-VP4 or eGFP-F52/70-VP4 expression plasmids, or a control plasmid expressing eGFP alone. Twenty-four hours post-transfection, cells were re-transfected with poly I:C. At 6 h post-transfection, cells were lysed and luciferase activity quantified. Firefly luciferase activity was normalized to Renilla expression. Data presented are the means of three independent experiments and passed a Shapiro-Wilk normality test before being analyzed by a one-way ANOVA and a Tukey's multiple comparison test (*$p < 0.05$, **$p < 0.01$). Error bars represent the standard error of the mean (SEM). In a parallel experiment, transfected cells were lysed and samples denatured and subject to SDS-PAGE gel electrophoresis followed by transfer to a nitrocellulose membrane and staining with anti-VP4 and anti-β-actin antibodies in a western blot.

The Ability of the UK661 VP4 Protein to Antagonize Type I IFN Responses Was Reduced in the Context of the Whole Virus *In vitro* and *In vivo*

In order to determine the extent to which strain dependent differences in the VP4 protein antagonized type I IFN responses in the context of a whole virus, chimeric viruses were generated with the VP4 gene from either the UK661 or F52/70 strains in the backbone of a highly attenuated cell culture-adapted vaccine strain, PBG98 (PBG98-VP4^{UK661} and PBG98-VP4$^{F52/70}$, respectively). DF-1 cells were infected with recombinant chimeric or wt PBG98 viruses (MOI 1) and RNA was extracted, reverse transcribed and the fold change in viral RNA quantified by qPCR at several time points post-infection. All viruses replicated to a maximum of 10^4-10^5 fold change in viral RNA per mL supernatant, with no significant differences in viral replication

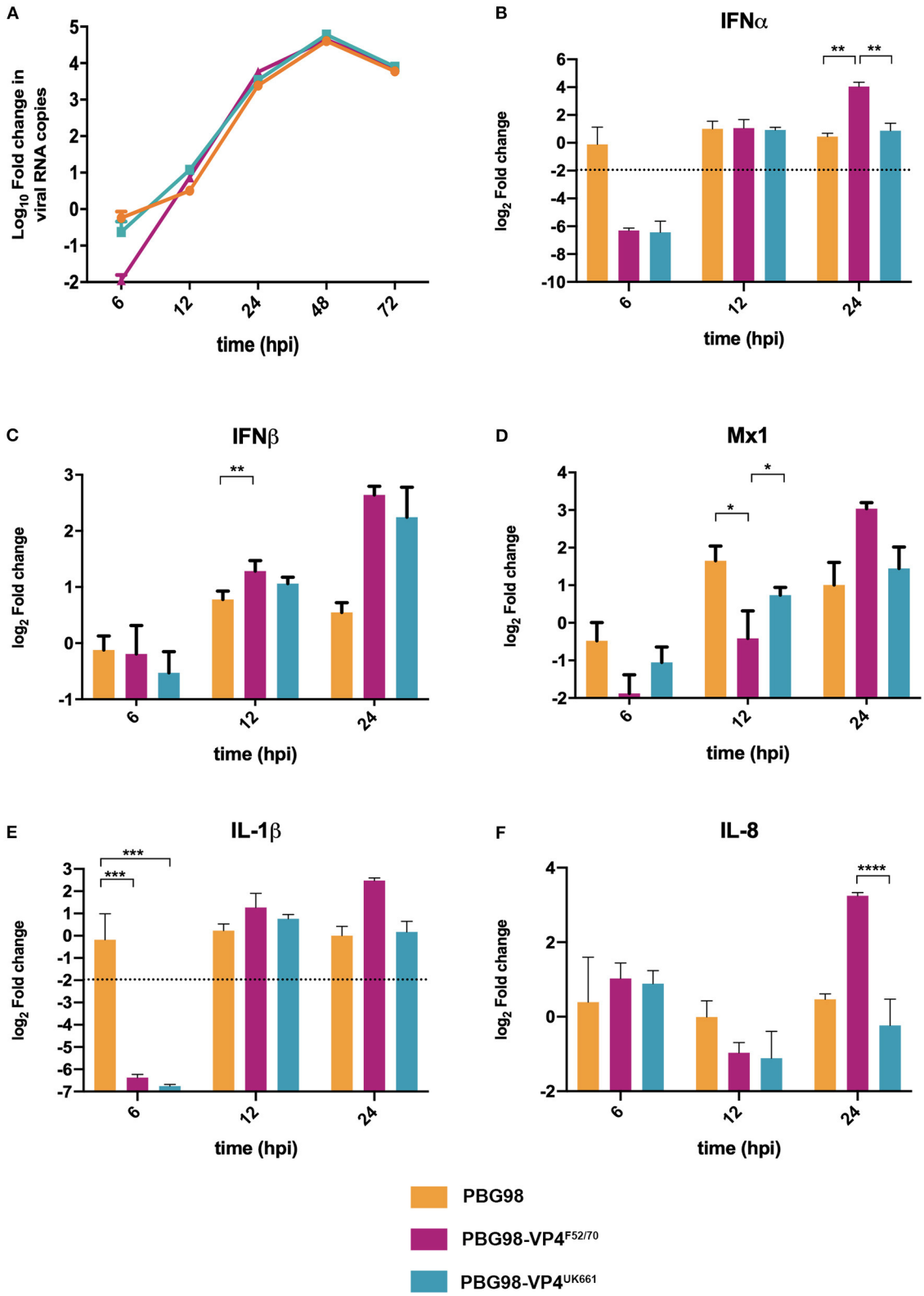

FIGURE 5 | The PBG98-VP4 ^{UK661} virus inhibits the induction of IFNα and IL-8 to a greater extent than the PBG98-VP4$^{F52/70}$ virus *in vitro*. DF-1 cells were infected with PBG98, PBG98-VP4^{UK661}, and PBG98-VP4$^{F52/70}$ viruses at an MOI of 1, before RNA was extracted at the indicated time points post-infection and reverse

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FIGURE 5 | transcribed. Virus specific primers were used to amplify the cDNA by quantitative (q)PCR, the CT values were normalized to the housekeeping gene RPLPO and the $\log_{10}$ fold change in virus gene expression was determined for the infected samples relative to the mock-infected controls in a ΔΔCT analysis and plotted. A one-way ANOVA and a Tukey's multiple comparison test was performed where no significant difference was found at any time point between the three viruses **(A)**. A panel of genes, IFNα **(B)**, IFNβ **(C)**, Mx1 **(D)**, IL-1β **(E)**, and IL-8 **(F)**, were amplified by qPCR using specific primer sets for target genes, before the CT values were normalized to the housekeeping gene RPLPO and the $\log_2$ fold change in gene expression determined for the infected samples relative to the mock-infected controls in a ΔΔCT analysis and plotted. Data are representative of at least three replicate experiments and passed a Shapiro-Wilk normality test before being analyzed by a one-way ANOVA and a Tukey's multiple comparison test ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$). The mean values are plotted and the error bars are the standard error of the mean (SEM). The dashed horizontal line represents the cut-off, below which genes were significantly down-regulated.

kinetics (**Figure 5A**). Consistent with our previous observations, the expression of IFNα was significantly reduced in cells infected with the PBG98-VP4^{UK661} virus compared to cells infected with the PBG98-VP4$^{F52/70}$ virus at 24 hpi (**Figure 5B**), and, although not statistically significant, the same trend was observed 24 hpi for IFNβ and Mx1 (**Figures 5C,D**). Interestingly, by 24 hpi, both chimeric viruses induced elevated IFNβ compared to the recombinant wt PBG98 virus. In addition, by 24 hpi, the PBG98-VP4^{UK661} virus induced a lower level of expression of IL-1β and IL-8 than the PBG98-VP4$^{F52/70}$ virus, which reached statistical significance for IL-8 ($^{****}P < 0.0001$). These data demonstrate that although the replication titers of the two chimeric viruses were similar, the expression of type I IFNα and pro-inflammatory cytokine IL-8 was lower during infection with the PBG98-VP4^{UK661} virus compared to the PBG98-VP4$^{F52/70}$ virus. This suggests that differences in the inhibition of innate immune responses is likely due to an intrinsic difference in the sequence of the VP4s, rather than due to different amounts of the protein being produced.

To compare virus replication kinetics and host gene expression *in vivo,* groups of 18 chickens were inoculated with chimeric and wt PBG98 viruses, or mock inoculated with buffer alone. At 2, 4 and 14 days post-inoculation, the BFs were harvested, RNA was extracted and the expression of IBDV, IFNα, IFNβ, Mx1, IL-6, IL-8, and IL-1β quantified by RTqPCR. There was no significant difference in viral replication between any of the groups (**Figure 6A**). However, virus replication was somewhat low (up to 10^3 fold change in viral RNA per gram of BF tissue), possibly due to the cell-culture adapted nature of the backbone. Consistent with our previous observations, at day 2 post-inoculation, the expression of IFNα was significantly lower in the BF of birds infected with the PBG98-VP4^{UK661} virus compared to the PBG98-VP4$^{F52/70}$ virus ($^*P < 0.05$) (**Figure 6B**). This trend was the same at day 4 post-inoculation although this did not reach statistical significance. Likewise, the average IFNβ expression at day 4 post-inoculation was lower in the BF of birds infected with the PBG98-VP4^{UK661} virus compared to the PBG98-VP4$^{F52/70}$ virus, but this did not reach statistical significance (**Figure 6C**). Mx1 expression was similar in birds infected with PBG98 and the chimeric viruses at days 2 and 4 post-inoculation (**Figure 6D**). Comparing the pro-inflammatory response between these viruses, IL-1β expression was significantly lower in the BFs of birds inoculated with either of the chimeric viruses at 2 days post-inoculation compared to recombinant wt PBG98 ($^{***}P < 0.001$) (**Figure 6E**), but there was no significant difference in IL-8 expression between any of the virus groups at day 2 or 4 post-inoculation (**Figure 6F**). Taken together, these data demonstrate that the chimeric virus containing the VP4 gene from the vvIBDV strain UK661 induced a lower level of type I IFNα compared to the chimeric virus containing the VP4 gene from the cIBDV strain F52/70 both *in vitro* and *in vivo*. However, the effect of the VP4 protein on IFNβ, Mx1, or pro-inflammatory cytokines was reduced in the context of virus infection, compared to ectopic expression.

DISCUSSION

Very virulent strains of IBDV emerged in the 1980s, causing up to 60% mortality in some commercial flocks (van den Berg et al., 2000). However, the molecular basis for this increased virulence remains poorly understood (Chettle et al., 1989; Nunoya et al., 1992). We have previously shown that the vvIBDV UK661 was able to down-regulate type I IFN and a selection of ISGs to a greater extent than a vaccine strain, D78, in primary B cells cultured and infected *ex vivo* (Dulwich et al., 2017). Here, we extend these observations by demonstrating that UK661 is also able to down-regulate type I IFN and pro-inflammatory cytokine responses compared to a classical field strain, and we confirm that this occurs not only *in vitro*, but also *in vivo* (**Figures 2**, **3**).

Other studies have compared strain-dependent differences in innate immune responses to IBDV infection, however, the majority have compared vv strains with cell culture adapted (ca) strains. For example, He et al. found TLR3, IL-8, and IFNβ expression were more up-regulated in response to the vvIBDV strain than a vaccine strain, and Liu et al. found elevated expression of cytokines following vvIBDV infection compared to a vaccine strain, however the vv strain replicated to significantly higher titers that the vaccine strains in both studies, making comparison of gene expression changes challenging (Liu et al., 2010; He et al., 2017). In contrast, in our study, there was no significant difference in peak virus replication between the F52/70 and UK661 strains, meaning that differences in gene expression are due to factors unrelated to viral load. Unfortunately, some studies comparing the innate immune response following vvIBDV infection to caIBDV strains also inoculated birds with different amounts of virus, making a direct comparison of gene expression difficult (Yu et al., 2015). To our knowledge, only one previous study, by Eldaghayes et al., has compared classical and vvIBDV strains *in vivo* (Eldaghayes et al., 2006). Our data are consistent with this work, which also reported that a vv IBDV strain induced reduced type I IFN responses compared to a classical strain. However, the authors conducted two separate *in vivo* studies, one with each virus, and did not compare the two viruses in the same study. Moreover,

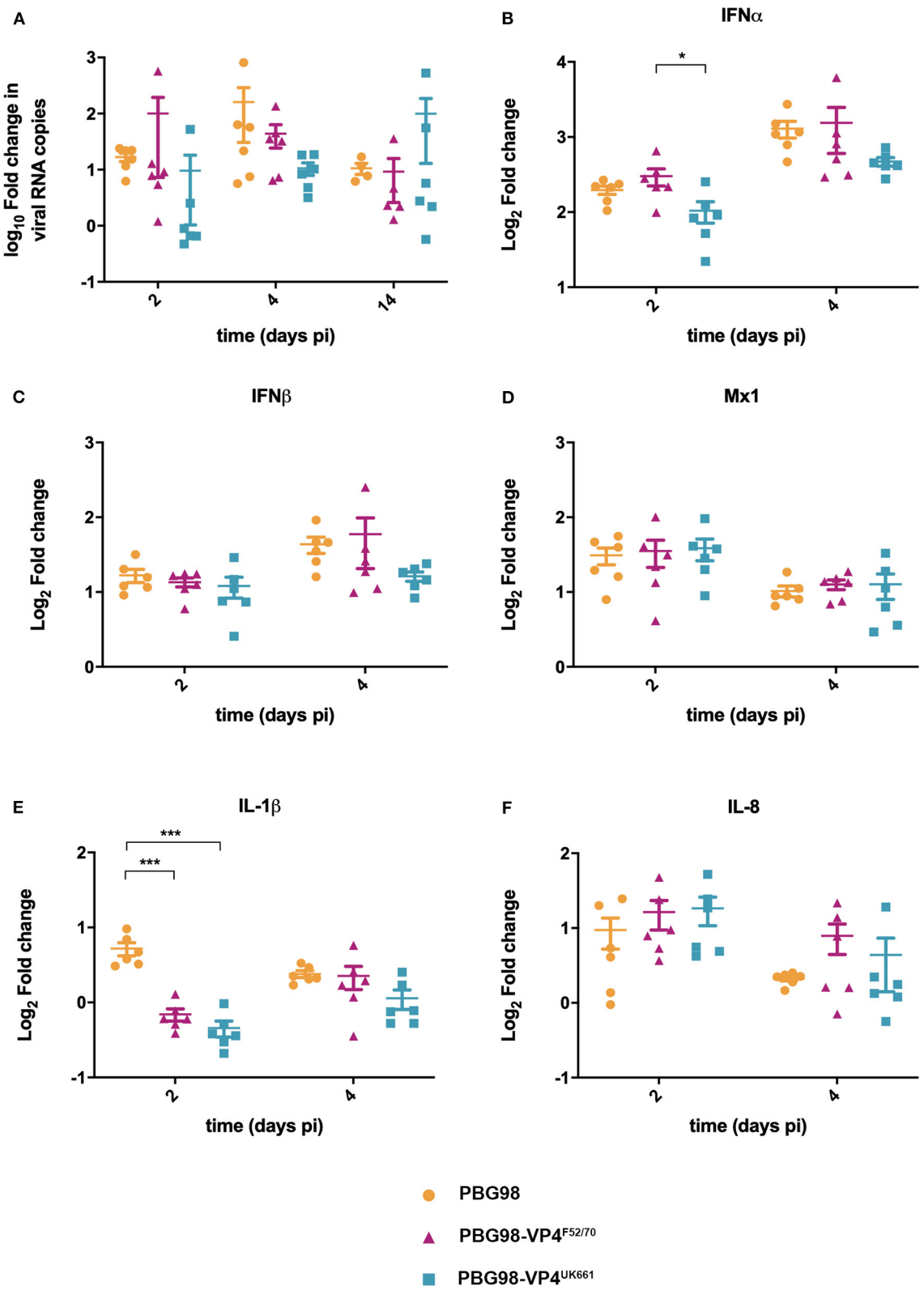

FIGURE 6 | The PBG98-VP4 ^{UK661} virus inhibits the induction of IFNα to a greater extent than the PBG98-VP4$^{F52/70}$ virus *in vivo*. Birds were inoculated with 1.8 × 10^3 $TCID_{50}$ of the PBG98, PBG98-VP4^{UK661}, and PBG98-VP4$^{F52/70}$ viruses, and the bursa of Fabricius was harvested at necropsy from 6 birds per group at 2, 4, and

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FIGURE 6 | 14 days post-inoculation. RNA was extracted prior to reverse transcription to cDNA and qPCR amplification with virus-specific primers. CT values were normalized to a housekeeping gene and expressed as log_{10}fold change viral RNA relative to mock-infected samples as per the ΔΔCT method. The data passed a Shapiro-Wilk normality test before being analyzed by a one-way ANOVA and a Tukey's multiple comparison test (not significant) **(A)**. At 2 and 4 days post-inoculation, cDNA was amplified by qPCR for a panel genes: IFNα **(B)**, IFNβ **(C)**, Mx1 **(D)**, IL-1β **(E)**, and IL-8 **(F)**. The CT values were normalized to the housekeeping gene RPLPO and expressed relative to mock-infected samples using the ΔΔCT method. Data are representative of at least three replicate experiments and passed a Shapiro-Wilk normality test before being analyzed by a one-way ANOVA and a Tukey's multiple comparison test (*$p < 0.05$, ***$p < 0.001$). Horizontal lines represent the mean and error bars represent the standard error of the mean (SEM).

birds were inoculated with a different dose of each virus, making a comparison of gene expression challenging. We extend these observations by directly comparing the vv and classical strains in the same *in vivo* study, in birds inoculated with the same dose of each virus.

We also demonstrate that the differences in IFN antagonism are, in part, due to strain-dependent differences in the VP4 proteins (**Figures 4–6**). The VP4 protein from vvIBDV strain Lx has previously been shown to act as an IFN antagonist through an interaction with the host glucocorticoid-induced leucine zipper (GILZ) protein (Li et al., 2013; He et al., 2018). GILZ plays a key role in the regulation of NF-κB activation by binding to the p65 subunit and preventing its translocation into the nucleus and the downstream expression of cytokines (Di Marco et al., 2007). The Lx VP4 protein has been shown to bind to GILZ, preventing its ubiquitination and degradation, resulting in its accumulation in the cytoplasm. Consequently, the VP4-GILZ interaction reduces p65 translocation into the nucleus, leading to a reduction in the expression of pro-inflammatory cytokines and type I IFN responses (He et al., 2018). We extend these observations by demonstrating that there are strain dependent differences in the extent to which VP4 antagonizes type I IFN induction. Elucidating differences in the mechanism of action of the different VP4 proteins was beyond the scope of this study, however, the amino acid sequence of the UK661 VP4 is the same as the Lx VP4, meaning that the UK661 VP4 will bind GILZ and inhibit NF-κB activation, whereas it is possible the affinity of the F52/70 VP4 for GILZ may be reduced. We speculate that the VP4 proteins of vv IBDV strains have a higher affinity for binding GILZ than the VP4 proteins of less virulent strains, meaning the vv IBDV strains may be able to inhibit NF-κB activation to a greater extent than less virulent strains. As NF-κB activation is partly responsible for the induction of antiviral type I IFN responses and pro-inflammatory cytokine responses, this model is consistent with our observations.

We demonstrated the involvement of the VP4 protein in the antagonism of type I IFN responses both by luciferase reporter assay, and the use of chimeric viruses expressing the VP4 protein from either UK661 or F52/70. However, the phenotype we observed with the chimeric viruses was less pronounced than that observed by luciferase reporter assay as, while the PBG98-VP4^{UK661} virus induced lower levels of IFNα, IFNβ, and Mx1 than the PBG98-VP4$^{F52/70}$ virus *in vitro*, this only reached statistical significance for IFNα at 24 hpi. This suggests that the VP4 gene is not the sole determinant of the difference in type I IFN responses observed between the UK661 and F52/70 viruses. Other IBDV proteins have previously been implicated in the inhibition of type I IFN responses, for example the VP3 protein is known to bind the dsRNA genome of IBDV and competitively inhibit detection by MDA5, preventing the downstream signaling of the IFN pathway and the production of type I IFN (Ye et al., 2014), and it is likely that multiple genes work in concert to produce the observed phenotypes.

When the chimeric viruses were inoculated into chickens, both replicated to low titers and caused few clinical signs, most likely because these viruses possess the backbone of a cell culture adapted, highly attenuated virus. We were therefore unable to ascertain whether strain-dependent differences in VP4 affected IBDV virulence. Previous studies, using chimeric viruses with segments A and B from strains of differing virulence, found that both segments contributed to virulence (Fernandez-Arias et al., 1997; Liu and Vakharia, 2004; Nouen et al., 2012; Escaffre et al., 2013). While the mechanism is not yet understood, the effect of VP1 mutations may be related to viral replication, whereas VP2 has been shown to activate apoptosis via the reduction of the anti-apoptotic molecule, ORAOV1 (Qin et al., 2017), and VP5 plays a key role in apoptosis by preventing it early during infection and by activating it at later time points (Lin et al., 2015). Virulence is therefore likely to be a complex phenotype, however, it is possible that the VP4 sequence could contribute to this. Five of the nine amino acid residues in UK661 that were different from F52/70 are also found in diverse vv IBDV strains from different geographical regions, but were not found in other classical or attenuated strains (31I, 170Y, 175N, 205S, and 241D) (**Supplementary Figure 3A**). It is therefore possible that these amino acids represent a VP4 "genetic signature of virulence." We speculate that the enhanced ability to antagonize innate immune responses is at least partly due to features of the VP4 from vvIBDV strains. By antagonizing antiviral type I IFN responses to a greater extent, the VP4 could enhance the replication of vv strains in the BF and lead to more pathology, and by antagonizing pro-inflammatory cytokine responses, the VP4 could hinder protective immune cell infiltration to the BF, further exacerbating pathology caused by the vv strains. Moreover, given that the classical strains emerged in the 1960s and vv strains subsequently emerged in the 1980s, it is tempting to speculate that vvIBDVs evolved to have a VP4 protein with an enhanced ability to inhibit NF-κB activation, promoting increased virus fitness due to suppressed antiviral responses. Consistent with this hypothesis, the replication of the UK661 virus was significantly enhanced in DT40 cells compared to the F52/70 virus (**Figure 3A**), although we did not see a difference in peak titer between the PBG98-VP4^{UK661} virus and the PBG98-VP4$^{F52/70}$ virus in DF-1 cells, possibly because innate immunity was not significantly affected until later in the replication cycle of the chimeric viruses for reasons that remain poorly understood.

The presence of the VP4 protein from the F52/70 IBDV strain in the cytoplasm of the host cell not only failed to inhibit

the induction of type I IFN, but actually triggered an innate immune response (**Figure 4**), although the molecular basis for this remains undetermined. It is possible that the VP4 protein from the UK661 strain either does not trigger the same pathways, or inhibits the induction of these responses. Consistent with this, the clinical scores of birds inoculated with the F52/70 virus were actually higher than birds inoculated with UK661 at 48 hpi (**Figure 1B**). At first this seems counter-intuitive that the vv strain would cause less severe symptoms than the classical strain, however, this may be due to the increased expression of type I IFN and pro-inflammatory genes in birds inoculated with the F52/70 virus, as stimulation of these innate immune responses would be expected to result in clinical signs such as lethargy, depression, and ruffled feathers as observed to a greater extent in the F52/70-inoculated group. This further underpins the complexity of defining virulence and highlights that our understanding of why some birds reach humane end points, whereas others do not, remains unknown.

Our study is not without limitations: Gene expression was quantified by RTqPCR, and only a small panel of genes were investigated. It would be beneficial in the future to compare gene expression by RNA-Seq to gain a more comprehensive comparison of strain-dependent differences in expression. Never-the less, our dataset does allow us to draw useful conclusions. Additionally, we only compared the UK661 strain with the F52/70 strain and it would be interesting to compare vv, classical, and vaccine strains, and possibly also diverse strains from different geographical regions, or serotype 1 compared to serotype 2. However, this was beyond the scope of the current project. Despite its limitations, our study does provide useful information that can be used to inform IBDV surveillance efforts and improve IBDV vaccines: Identifying genetic signatures of increased IBDV virulence could be used to better inform national surveillance efforts in order to calculate the potential threat of an emerging strain as early as possible. Moreover, identifying genetic signatures of attenuation could be used to engineer a rationally designed live vaccine candidate. For example, it might be beneficial to explore the potential, as novel vaccine candidates, of chimeric viruses engineered with the VP4 gene from attenuated strains in the backbone of a field strain.

Taken together, our data demonstrate that UK661 induced the expression of lower levels of anti-viral type I IFN responses than the classical strain *in vitro* and *in vivo* and this was, in part, due to strain-dependent differences in the VP4 protein. We speculate that this might enhance viral fitness and contribute to the enhanced virulence of UK661. This provides new information that could be used to improve IBDV surveillance efforts and control strategies.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/ **Supplementary Material**.

ETHICS STATEMENT

All animal procedures conformed to the United Kingdom Animal (Scientific Procedures) Act (ASPA) 1986 under Home Office Establishment, Personal and Project licenses, following approval of the internal Animal Welfare and Ethic Review Board (AWERB) at The Pirbright Institute.

AUTHOR CONTRIBUTIONS

KD performed experiments, analyzed data, and wrote the first draft of the manuscript. AA and AG performed experiments. EG and MS helped to analyse data and edited drafts of the manuscript. AB conceptualized the study, obtained funding, helped to conduct experiments and analyse data, and edited drafts of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.00315/full#supplementary-material

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frontiers in Cellular and Infection Microbiology

REVIEW
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A Testimony of the Surgent SARS-CoV-2 in the Immunological Panorama of the Human Host

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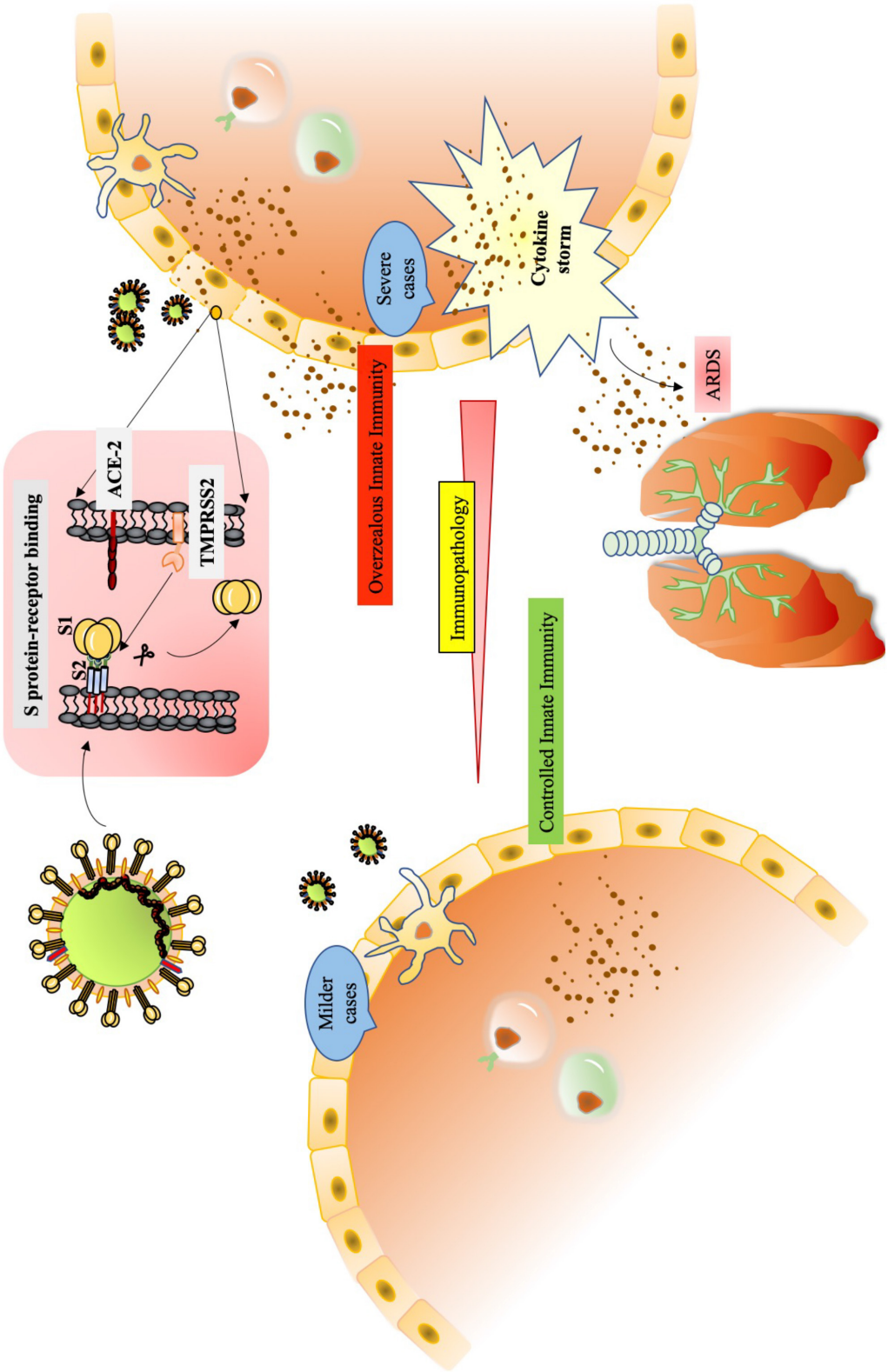
The resurgence of SARS in the late December of 2019 due to a novel coronavirus, SARS-CoV-2, has shadowed the world with a pandemic. The physiopathology of this virus is very much in semblance with the previously known SARS-CoV and MERS-CoV. However, the unprecedented transmissibility of SARS-CoV-2 has been puzzling the scientific efforts. Though the virus harbors much of the genetic and architectural features of SARS-CoV, a few differences acquired during its evolutionary selective pressure is helping the SARS-CoV-2 to establish prodigious infection. Making entry into host the cell through already established ACE-2 receptor concerted with the action of TMPRSS2, is considered important for the virus. During the infection cycle of SARS-CoV-2, the innate immunity witnesses maximum dysregulations in its molecular network causing fatalities in aged, comorbid cases. The overt immunopathology manifested due to robust cytokine storm shows ARDS in severe cases of SARS-CoV-2. A delayed IFN activation gives appropriate time to the replicating virus to evade the host antiviral response and cause disruption of the adaptive response as well. We have compiled various aspects of SARS-CoV-2 in relation to its unique structural features and ability to modulate innate as well adaptive response in host, aiming at understanding the dynamism of infection.

Keywords: SARS-CoV-2, receptor binding domain (RBD), angiotensin-converting enzyme 2 (ACE-2), transmembrane protease serine 2 (TMPRSS2), acute respiratory distress syndrome (ARDS), cytokine storm, inflammatory cytokines, innate immunity

INTRODUCTION

The ever-increasing number of perilous alterations of wildlife through various human interventions have presented the global face of humanity with growing experiences of several infectious outbreaks. A protracted meeting between humans and bats has presented a timeline of pandemics caused by the coronaviruses (Cui et al., 2007; Fan et al., 2019; Sifuentes-Rodríguez and Palacios-Reyes, 2020). History now tells of the 2002–2003 Severe Acute Respiratory Syndrome (SARS) and 2011 Middle East Respiratory Syndrome (MERS), both being zoonotically originated infections in humans that were caused by new coronaviruses, SARS-CoV and MERS-CoV, respectively (Minakshi et al., 2014; de Wit et al., 2016; Song et al., 2019; Prompetchara et al., 2020; Rabaan et al., 2020). A new addendum to this is the late 2019 outbreak due to another novel coronavirus, SARS-CoV-2, that caused illness related to the respiratory system (Guo et al., 2020). The emergence



Graphical Abstract | Pathophysiology of SARS-CoV-2.

of SARS-CoV-2 is putting tremendous pressure on the international community wherein we are witnessing the unprecedented lockdown imposed in various countries across the globe.

KNOWING SARS-CoV-2

The late December of 2019 witnessed cases of respiratory infection symptoms like cough, fever and dyspnea that led to pneumonia among clusters of patients in Wuhan (China). The condition was intriguing as the causative agent was unknown thus driving the medical community to search for the reason (Zhu et al., 2020). This investigation led to the isolation of a "novel" coronavirus, which was identified through next-generation sequencing and given a provisional name, the 2019 novel coronavirus (2019-nCoV) (Lu et al., 2020).

Out of the four genera of coronaviruses, α, β, γ, and δ, the human coronaviruses (HCoVs) fall in the genus α (HCoV-229E and NL63) and the genus β (MERS-CoV, SARS-CoV, HCoV-OC43, and HCoV-HKU1) (Perlman and Netland, 2009; Halaji et al., 2020). The genus β comprises of five subgenera where the entire bat derived coronaviruses fall. Further phylogenetic analysis showed that this 2019-nCoV hailed from the bat coronavirus reservoir, although during transmission to human, an intermediate host between bat and human is suggested (Lam et al., 2020; Lu et al., 2020). Being a β-coronavirus, the isolate was 88% identical to coronaviruses of bats that cause SARS (bat-SL-CoVZC45 and bat-SL-CoVZXC21), 82% identical to SARS-CoV and 50% identical to MERS-CoV (Cascella et al., 2020; Lu et al., 2020). The International Virus Classification Commission named it the SARS-CoV-2 (Li X. et al., 2020).

The SARS-CoV-2 has round to elliptical form (diameter range 60–140 nm) that often displays pleomorphy. Representing a typical coronavirus genome, the SARS-CoV-2 has a non-segmented, positive sense, single-stranded RNA (Cascella et al., 2020; Coronaviridae Study Group of the International Committee on Taxonomy of Viruses, 2020; Zhu et al., 2020). Though the phylogenetic analysis presented data supporting the similarity of SARS-CoV-2 with β-coronaviruses of bats, the new virus showed itself to be distinct from the previously known SARS-CoV and MERS-CoV. The conserved ORF1ab (replicase complex) was found to be <90% identical to other members of β-coronavirus (Zhu et al., 2020). Some of the genes of the SARS-CoV-2 shared <80% sequence identity to those of the SARS-CoV, but the sequence of amino acids in the seven conserved domains of replicase enzyme (ORF1ab) of SARS-CoV-2 showed 94.4% identity with that of SARS-CoV (Zhou P. et al., 2020). This finding ascertained that SARS-CoV-2 and SARS-CoV are belonging to the same species (Zhou P. et al., 2020).

THE OVERWHELMING POWER TO INFECT: ANALYZING THE CAPABILITIES OF SARS-CoV-2

Repurposing its existence, the SARS-CoV-2 jumped from animal to human and then human to human through droplets from patient's sneeze or cough as well as through direct physical contact (Chang et al., 2020; Li Q. et al., 2020). In no time the virus spread rapidly to establish infection not only through symptomatic but also through asymptomatic carriers, SARS-CoV-2 exhibited high potential to be the cause of a pandemic (Chang et al., 2020; Munster et al., 2020). Studies in support of this outbreak being in a very high-risk category with regard to its spreading, have estimated the transmissibility of SARS-CoV-2 in terms of R_0, the basic reproduction number. $R_0$is the representative value of the average number of new infections caused by one infected patient in a population. For $R_0 < 1$, an infected patient spreads virus to <1 person and this would lead to a fall in the strength of infection. Whereas, if $R_0 > 1$, the contagion will be transmitted to more than 1 person and the outbreak will increase. The value of R_0 is central to an infectious disease epidemiology (Liu et al., 2020). The estimated R_0 of SARS-CoV-2 ranges from 2.24 to 3.58 while that of SARS-CoV and MERS-CoV ranges from 2 to 5 and 2.7 to 3.9, respectively (Zhao S. et al., 2020). SARS-CoV-2 was isolated from Vero E6 and Huh7 cells where cytopathogenic effects were clearly shown (Zhou P. et al., 2020).

This review deals with the very novelty of the SARS-CoV-2 based on the reports the features of viral architecture, its mode of action and host response.

UNIQUE FEATURES IN THE SARS-CoV-2 GENOME

The genome organization of SARS-CoV-2 is discussed in **Figure 1A**. The 29,903 nucleotides RNA encodes structural proteins like spike protein (S), envelope protein (E), membrane protein (M), nucleocapsid protein(N), and accessory proteins, 3a, 6, 7a, 8, and 10 (Kim et al., 2020). The most alterable sequences in the coronavirus genome are harbored in the receptor-binding domain (RBD) of the S gene (Zhou P. et al., 2020). The *S* gene of SARS-CoV-2 has been studied to be longer than other reported SARS related coronaviruses (SARSr-CoV) (Zhou P. et al., 2020). This guided for the development of qPCR-based detection methods where the RBD, being the most variable sequence, was targeted (Zhou P. et al., 2020). Besides these data on RNA sequences, some significant differences in the amino acids of SARS-CoV and SARS-CoV-2 have been observed. The 8a protein was absent in SARS-CoV-2, the length of amino acids in 8b protein of SARS-CoV was 84 whereas that in SARS-CoV-2 was 121. The 3b protein of 154 amino acids in SARS-CoV was longer than that of SARS-CoV-2 displaying 22 amino acids (Wu et al., 2020). Worth noticing is the receptor-binding motif (RBM) in RBD that doesn't have any substitutions whereas other sequences of RBD harbor six mutations (Wu et al., 2020). Amino acid substitution study in other genes of SARS-CoV-2 found two important substitutions in NSP2 and NSP3 at the respective positions of 61 and 102 when compared with that of SARS-CoV (Wu et al., 2020).

These aspects merit further studies to understand the novel infectious capacity of SARS-CoV-2.

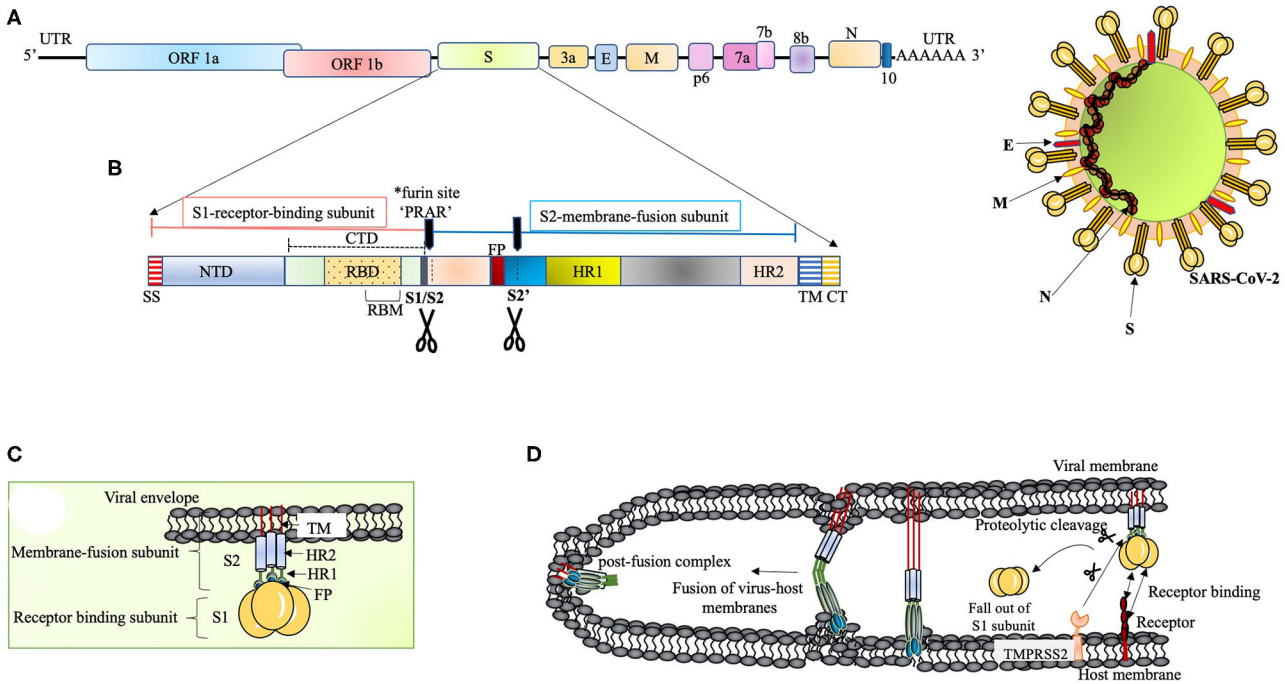


FIGURE 1 | (A) The proposed genome organization of SARS-CoV-2. The RNA has 5' UTR followed by the predicted ORF 1ab encoding polyprotein ab (pp1ab) for 16non-structural proteins (NSP1 to NSP10 and NSP12 to NSP16). This segment is succeeded by: Spike glycoprotein (S), ORF3a, Envelope protein (E), Membrane protein (M), ORF6, ORF7a, ORF7b, ORF8, Nucleocapsid protein (N) and ORF10 (Kim et al., 2020). The ORF1ab polyprotein has site for (Bagdonaite and Wandall, 2018) cleavage by the virus coded 3CLpro and PLpro to form RNA-dependent RNA polymerase (RdRp) and helicase (Anand et al., 2003; Srinivasan et al., 2020). **(B)** The S polypeptide. The S1, receptor binding subunit, shows NTD and CTD differentiation where CTD harbors RBD with an internal RBM, which is conserved and recognizes ACE2. The S2, membrane fusion subunit, has fusion peptide (FP), S2' proteolytic site, two heptad-repeats, HR1 and HR2, and a transmembrane domain followed by cytoplasmic tail (CT) (Coutard et al., 2020). The S polypeptide is processed by extracellular proteases at S1/S2 site at the time of infection. The S protein has acquired a polybasic (*furin) site for cleavage at S1/S2 boundary, which is a unique feature of SARS-CoV-2 (Andersen et al., 2020; Walls et al., 2020). This feature widens the cell tropism of the virus (Kido et al., 2012). The polybasic site (PRRA) has a characteristic proline lead that has been predicted to invite addition of *O*-linked glycans (Andersen et al., 2020). **(C)** The S protein. The S protein (~1,200 aa long) is homotrimeric type I transmembrane protein. The S1/S2 cleavage site is predicted to acquire addition of *O*-linked glycans on the turn made by proline residue (PRRA) at the furin site. *O*-linked glycans have been studied to mask the immunodominant epitopes of viral antigens from immune recognition and augment virus-cell fusion through conformational changes (Bagdonaite and Wandall, 2018). **(D)** Proposed scheme of S protein and host receptor interaction. The S protein, upon binding with the host cell receptor, undergoes a step of priming wherein the extracellular proteases cleave off S1 subunit. This is followed by conformational changes in S2 subunit with the intercalation of FP within the host membrane. Finally, the HR1 and HR2 fold back to give post-fusion complex.

THE VIRAL ENTRY INTO THE CELL

The S protein of SARS-CoV-2 docks on the host cells by recognizing the receptor protein (a zinc-dependent carboxypeptidase), angiotensin-converting enzyme 2 (ACE2) as did the SARS-CoV (Li et al., 2003; Gheblawi et al., 2020; Zhang H. et al., 2020). ACE2 not only functions in the regulation of blood pressure but also against severe acute lung failure. The expression profile of ACE2 is wide. Apart from lungs, the other organ epithelial cells showing high expression levels of ACE2 are: bone marrow, brain, mouth, salivary glands, nasal lining, heart, thyroid, adipose tissue, gastrointestinal tract (duodenum, small intestine, colon, rectum), gallbladder, adrenal glands, kidneys, and male genital tissues (seminal vesicles and testis) (Li et al., 2020).

In the case of SARS-CoV, it was shown that the degree of lung injury was directly linked to ACE2 downregulation (Imai et al., 2005; Kuba et al., 2005; Sarzani et al., 2020). Studies have shown that the human ACE2 gets engaged with the S glycoprotein of SARS-CoV-2 with comparable degree of affinity as in the case of SARS-CoV. The sequence substitution study in RBD of SARS-CoV-2 discussed earlier, hinted that the interface for interaction with the host must be playing a crucial role in the viral tropism (Wu et al., 2020; Zost et al., 2020).

The S polypeptide and glycoprotein are discussed in **Figures 1B,C**, respectively. RBDs are the major determinants of host range and capacity to cross species barrier in coronaviruses (Li, 2015). It is therefore relevant to discuss NTD of the S1 subunit. It was proposed that coronaviruses stealthily inserted galactin gene (host lectin) in their S1 NTD, which resulted in their evolutionary divergence (Li, 2015). Conferred with the power of varying sugar-binding capacity, these viral lectins find their location in the cavities of the spike subunit whereby they can escape host antibodies during infection (Li, 2015). The S2 subunit (fusion-catalyzing domain, FDs) is the membrane-anchored component with necessary fusion machinery. After S1 undergoes

proteolytic cleavage, the FDs get revealed through irreversible conformational transformations resulting in the intercalation of fusion peptide into the host membrane (Park et al., 2016) (**Figure 1D**). The presence of extensive N-linked glycans in the homotrimers of S protein not only help in gaining access to the host proteases for cleavage required for fusion but also attract neutralizing antibodies (Belouzard et al., 2009; Millet and Whittaker, 2014; Walls et al., 2017).

A concerted role of various host factors in the cell tropism of the virus is very important. The entry of SARS-CoV-2 into the cell is largely dependent on host protease activity, wherein the transmembrane protease, serine 2 (TMPRSS2), co-expressed with ACE-2 in the lung tissue, has been shown to facilitate the processing of the S protein (Matsuyama et al., 2005, 2010) (**Figure 1D**). The cleavage of the S protein by TMPRSS2 has been shown in augmenting SARS-CoV pathogenesis (Glowacka et al., 2011; Reinke et al., 2017; Hoffmann et al., 2020). Acting as a double-edged sword for the virus, this feature not only aids in the cleavage of S protein to facilitate virus entry but also interferes with neutralizing antibodies (Glowacka et al., 2011). This mode of direct entry through fusion with the host membrane was shown to be 100 times more effective than the endosome-mediated pathway of virus entry in SARS-CoV tropism (Matsuyama et al., 2005). Furthermore, the S protein has also been shown to associate with the C-type lectin expressed on the dendritic cells (DCs), where SARS-CoV infection is mediated *in trans* (Lau and Peiris, 2005).

HARKENING THE IMMUNOLOGICAL PAST TO DECIPHER SARS-CoV-2

Nearly two decades back, the upsurge of SARS-CoV and then latterly on MERS-CoV, has provided a plethora of information on the pathophysiology and pathogenesis of the ever-evolving human coronaviruses. Many studies are emerging where labs are using the data acquired through previous research on SARS-CoV for understanding the behavior of SARS-CoV-2. The pathogenesis of SARS-CoV involves a complex network of events that not only manifests as severe injury to lungs but also a widened effect to other organs of the body. The immunological evaluations done on SARS-CoV clearly underline situations like high viral load, a storm of cytokines like CCL3/MIP-1α, CXCL10/IP-10, and CCL2/MCP-1, substantial infiltration of lung by macrophages and monocytes and the very fast diminishing levels of T cells (Chen et al., 2010). The condition of hypoxemic respiratory failure manifesting as acute respiratory distress syndrome (ARDS), is majorly contributing to mortality in SARS-CoV-2 infection. ARDS has been characterized as a systemic inflammation where bilateral involvement of lungs and other organs is evident. The modality of ARDS displays massive build-up of inflammatory cytokines like IL-1β, IL-6, TNF-α etc. in both broncho-alveolar lavage fluid (BALF) and plasma circulation (**Figure 2**). A non-resolving inflammation sets in where polarization of monocytes/macrophages is observed with the production of nitric oxide (NO), ROS and inflammatory cytokines that exert damaging effects on the lungs (Liu et al., 2019).

Here we present evidences of the multipronged immunological response during SARS-CoV and MERS-CoV pathogenesis and bring out its correlation with the work done on the present SARS-CoV-2 behavior.

THE INNATE DEFENSE

The working of innate immunity starts with the detection of the pathogen-associated molecular patterns (PAMP) (viral component or intermediates of replication) by the pattern recognition receptors (PRRs), wherein the Toll-Like Receptors (TLRs), the NOD-like receptors (NLRs) and the RIG-I-Like Receptors (RLRs) represent as significant PRRs against RNA viruses. The PRRs are ubiquitously distributed on the plasma membrane, endosomal membranes and in the cytosol (Arpaia and Barton, 2011; Rathinam and Fitzgerald, 2011). The sensing of viral PAMPs by these cellular arsenals (PRRs) charges a battery of programming that involves gene expression upregulation of inflammatory antivirals like cytokines, chemokines and interferons (IFNs) (Totura and Baric, 2012). These molecules being the "whistle blowers," pave way for two important events: firstly, IFN-I induces a signaling network, where numerous IFN-stimulated genes (ISGs) are expressed and secondly the production of cytokines and chemokines stimulate neutrophils, macrophages, NK cells and DCs. In an infected lung, there is copious number of macrophages and DCs that function in controlling the infection via the production of cytokines and acting as antigen presenting cells (APCs). Being first respondents to the pathogenic invasion, their interaction with the virus becomes a significant parameter in the conclusion of infection. The maturation of DCs, stimulated by inflammatory cytokines like tumor necrosis factor alpha (TNF-α) and IL-1, is very important event in the immune response of a host during a viral infection. The priming of T cells by the DCs in the lymphatic organs becomes the ensuing event before a series of immune responses set in. Therefore, incapacitation of DCs becomes an excellent target for the virus whereby the very early step of immune response initiation is overcome during early infection cycle of the virus. The antigen presentation by APCs on their major histocompatibility complex (MHC class I) or precisely human leukocyte antigen (HLA) complex and then their recognition by virus-specific CD8+ cytotoxic T lymphocytes (CTLs) is a hallmark step in the initiation of host immune response.

The bystander effect of IFN-I on the unaffected cells neighboring infected cells, provides significant antiviral status, recruits innate immune cells and primes the adaptive immune response. The activation of the JAK/STAT pathway by IFN-I robustly regulates immune response network. Additionally, stimulation of DCs, NK cells and CTLs are also key to IFN signaling (Cristiani et al., 2020). All these molecules not only orchestrate the local fight against the virus but also the adaptive immune response. But situation specific cross-talks between DCs and NK cells could lead to the amplification in innate response strength (Andoniou et al., 2005).

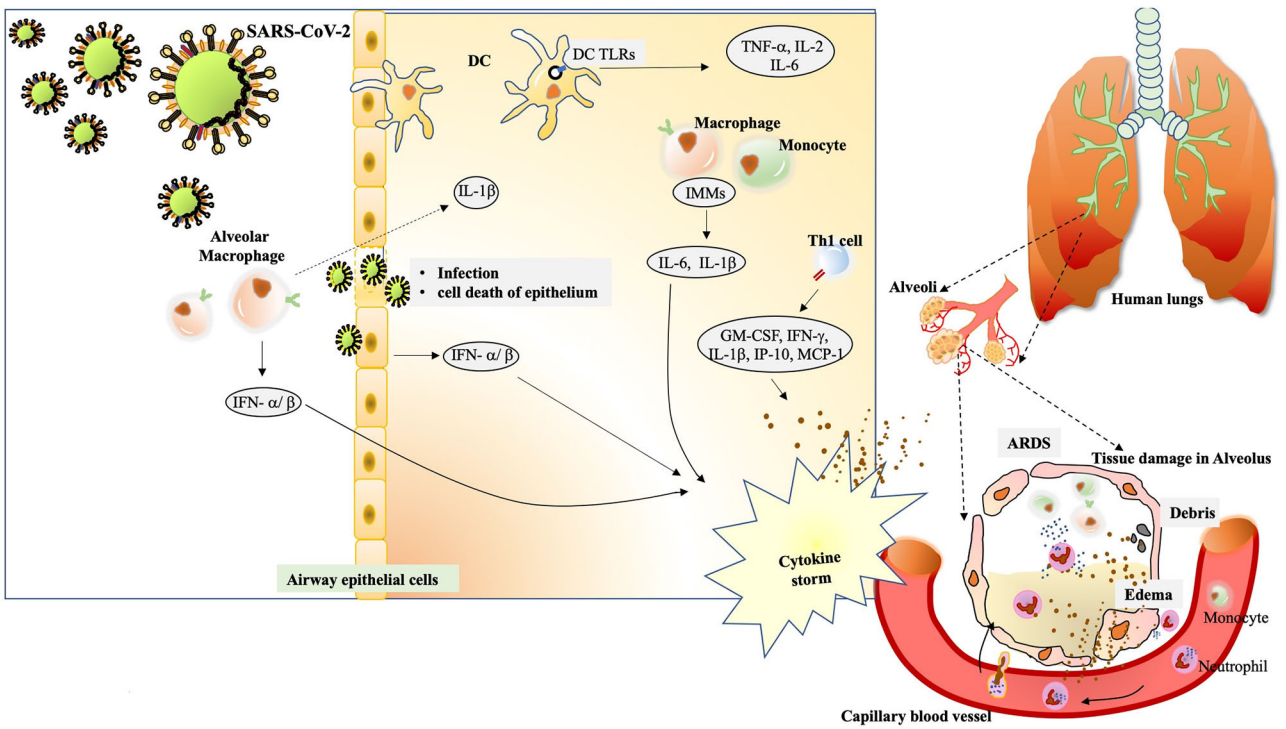


FIGURE 2 | The immunological drama mastered by SARS-CoV-2. The alveolar macrophages act as first sentinels in the airways. The innate arm of immunity shows overzealous activation during severe cases of SARS-CoV-2.After confronting SARS-CoV-2, macrophage inflammasome-mediated release of IL-1βinduces a robust host response visible during immunopathology. At the same time, they induce vigorous expression of IFN-α/β that add on to the cytokine storm. Inside the alveolus, the DCs also stimulate expression of TNF-α, IL-2 and IL-6 through TLR signaling. The cytopathic effect of SARS-CoV-2 can be seen in the epithelial damage. Also, the infected cells, exerting their "by stander" effect, also induce IFN-α/β. Subsequent interaction of virus/antigens with IMMs further augments in the concentration of inflammatory cytokines. Th1 activation also charges a set of cytokines to add up to cytokine storm. The cytokine storm exerts damaging effect in alveolar tissue. The extravasation of circulating neutrophils and monocyte under the existing cytokine stimulation manifests in edema and deposition of debris in the alveolus. The condition of ARDS is conspicuously registered.

THE ADAPTIVE DEFENSE

The innate arm of immunity is insufficient in not only clearing a posing infection but also registering memory for subsequent infections in the host (Flynn et al., 1998). The innate immune network passes on a set of instructions to the adaptive network, after deciphering the "non-self" from the "self" (Janeway, 1989). The convention of adaptive immune response begins with the uptake and sampling of the virus/its antigens by DCs, which further mature into APCs and migrate to draining lymph node system to educate T and B cells (Grayson, 2006). After developing in the bone marrow, immature DCs translocate to the peripheral tissues like lungs, where they gather antigens with the help of PRRs and get stimulated by several pro-inflammatory cytokines resulting in their maturation. The event marks down-regulation of PRRs, showing up of MHC, CCR7, CD80, and CD86 on their cell surfaces (Saeki et al., 1999).

The cell mediated immunity involves the action of CTLs, NK cells and macrophages. The recognition of viral antigens stimulate the expression pattern of various cytokines by T helper (Th) cells. The T-dependent activation of B cells for the production of antibodies against virus, is facilitated by the CD4+ T cells (Xu and Gao, 2004). The effects of CD8+ T cells are exerted in two ways: secretion of cytokines (IFN-γ, TNF-α, IL-2) as well as chemokines and cytolysis of the target cells (Frasca et al., 1998). The Ths, Th1 cells direct the activation of macrophages and Th2 cells stimulate eosinophils. On the other hand, the B cells, through their antibodies, regulate the complement pathway, phagocytosis and degranulation of mast cells.

The very critical action of CD8+ T cells in clearing the virus-infected cells, has been well-studied in mice (Doherty et al., 1997; Topham et al., 1997). The physiological role of CTLs in combating respiratory virus infection is also translated through the lysis of infected cells leading to tissue destruction (during eclipse phase of virus replication) (Zinkernagel and Althage, 1977). The condition of immunopathology is worsened in cases of cytopathic viruses where tissue damage is collaterally executed by CTL response to the virus (Reviewed in Zinkernagel, 1989).

The Behavior of SARS-CoV

The SARS cases showed high serum concentrations of cytokines, neutrophils, and monocyte-macrophages in the lungs. This trend of extensive immunopathology arising out of severe inflammation has been studied to be a notable feature of other coronaviruses (Kindler and Thiel, 2016). In the context of

HLA alleles, HLA-A24 being the most common HLA-A alleles, has been studied to be predominant in Eastern Asiatic region (Middleton et al., 2003). In the case of SARS-CoV, the HLA-A2-restricted CTL epitopes have been studied (Tsao et al., 2006; Kohyama et al., 2009; Ohno et al., 2009). The immunogenic region of N protein, which is an HLA-A*2402 restricted epitope, has also been studied to be a prominent candidate CTL antigen in the case of SARS-CoV (Liu et al., 2010). Previous studies have given a range of polymorphism in HLA in relation to susceptibility and protection from the SARS-CoV infection (Keicho et al., 2009; Wang et al., 2011).

Studies on SARS-CoV infected DCs have been of prime importance here. DCs are known to express a variety of receptors for pathogen recognition where TLRs of two kinds, the one present on the cell surfaces (TLR-1, TLR-2, TLR-4, TLR-5, TLR-6, TLR-10) and those expressed within intracellular compartments (TLR-3, TLR-7, TLR-8, TLR-9) are significant. The signaling cascade activation through TLRs results in DC induced production of costimulatory molecules like CD80 and CD86 as well as pro-inflammatory cytokines like TNF-α, IL-2, and IL-6 (Thoma-Uszynski et al., 2000; Kaisho and Akira, 2001). The signaling of TLR-7 in plasmacytoid DCs during SARS-CoV infection is antagonized by the Orf1a encoded PLPro (Papain-Like Protease) resulting in the diminishing of cytokine production and this acted against the establishment of antiviral response (Li et al., 2016). Other cellular targets antagonized by various SARS-CoV proteins are discussed in **Table 1A**. The activation of TLR-3 and TLR-4 through TRIF adapter protein imposes strong cell-intrinsic defense response against SARS-CoV infection (Totura et al., 2015). The use of TLR agonists have been well-projected as respiratory vaccine adjuvants (Zhao et al., 2012; Shirey et al., 2013; Perez-Giron et al., 2014). This prompted the usage of TLR-3 and TLR-4 agonists to have protection against SARS-CoV infection (Totura et al., 2015).

In the murine model of SARS-CoV, an increasing viral replication showed delayed response of IFN-I. The report showed milder or subclinical SARS in younger infected mice whereas aged mice developed a more severe version of the disease. This simulation was parallel to the development of SARS observed in humans where being "aged" guaranteed a severe form of the disease. IFN-I was not detected in BALF of mice until 24 h post infection, which potentiated the buildup of inflammatory monocyte-macrophages that release IL-6, IL-1β. This results in higher levels of cytokines, leakage in vasculature and abrogation in T cell response (Channappanavar et al., 2016). The serum build-up of inflammatory cytokines like IL-1β, IL-6, IL-12, Th1 cytokine, IFN-γ, and chemokine IP-10 were reported in SARS patients with overt pulmonary inflammation and lung damage. Additionally, there were substantial elevation of neutrophil chemokine IL-8 and monocyte chemoattractant protein-1 (MCP-1) (Wong et al., 2004). All of these observations unequivocally supported the overzealous innate response with massive infiltration of monocytes-macrophages and neutrophils in the SARS-CoV infection.

The intranasal administration of IFN prior to the peak reached during replication of virus displayed protective role in mice (Channappanavar et al., 2016). Paradoxically, IFN-I has been indicated to perpetuate fatal immunopathology by subverting the T cell response through inhibitory molecules like PD-1 and LAG-3. The study further attested that the lethality of SARS was not only contributed by TNF but also by the accumulation of IMMs (Channappanavar et al., 2016). Additionally, virus encoded proteins or the components of their replication have been well-documented as antagonists in the induction of IFN signaling. In this vein, SARS-CoV has been known to code proteins antagonizing RLR signaling (Shi et al., 2014).

The pro-inflammatory cytokines and chemokines produced in SARS-CoV infection were significant in numbers. Higher serum concentrations of TNF-α, IL-6, CCL3, CCL5, CCL2, CXCL10 were found in severe SARS patients (Channappanavar and Perlman, 2017). On the contrary, the levels of anti-inflammatory cytokines, IL-10 were low in severe cases of SARS-CoV (Chien et al., 2006).

The immunogenicity of the S protein showing the T cell epitopes were recognized for eliciting T cell response through the IFN-γ expression, in the convalescent SARS-CoV patients (Wang B. et al., 2004; Wang Y. D. et al., 2004). Alongside the S protein, the N protein of SARS-CoV also elicited persistent levels of anti-N antibodies and CD8+T cell response (Peng et al., 2006). The T cell mediated immune response against the N protein of SARS-CoV led to the generation of a strong population of IFN-γ producing T cells in animal studies (Zhao et al., 2005). The adverse cases of SARS-CoV infections showed lymphopenia with characteristic decline in both CD4+ as well as CD8+ T cells. However, there was a dramatic restoration of CD4+, CD8+ T cells, and B cells in patients who recuperated from SARS-CoV infection (Li et al., 2004).

The remarkable significance of virus-specific T cell mediated response was exemplified in the mouse model where a poorly stimulated immune system was shown to recover through the delivery of epitope-specific cultured CD8+ T cells (Zhao and Perlman, 2010).

The active role of humoral immunity is well-reported during SARS-CoV infection where the serum IgG, IgM, and IgA against viral antigens have been observed (Hsueh et al., 2004). The

TABLE 1A | Cellular targets antagonized by various SARS-CoV protein.

SARS-CoV	Proteins of the virus	Cellular targets	References
1.	Orf3b	Direct inhibitor of IFN-β	Spiegel et al., 2005
2.	Orf3a	Suppression of IFN signaling (PERK pathway)	Minakshi et al., 2009
3.	Orf6	Inhibits IFN signaling by interfering STAT1	Frieman et al., 2007; Siu et al., 2009
4.	M and N	Block IFN-I production and NF-κB signaling	Kopecky-Bromberg et al., 2007; Siu et al., 2009
5.	NSP1	Inhibit IRF3-STAT1 and NF-κB pathways	Wathelet et al., 2007
6.	NSP3	Antagonizes IFN-β	Frieman and Baric, 2008

S protein has emerged as a strong candidate for immune protection through vaccination. The antigenic potential of the N protein is very high in SARS-CoV as seen in the convalescent sera of the patients (Zhong et al., 2005). The S1 domain of S protein has been tested to stimulate neutralizing activity against monoclonal antibodies (Sui et al., 2004). Nonetheless, the development of ARDS in SARS-CoV patients coincided with IgG seroconversion (Peiris et al., 2003). The presence of higher neutralizing antibodies (Nab) against S protein (IgG) and more binding antibodies against N protein, was recorded in recovered patients of SARS-CoV (Zhang et al., 2006; Seydoux et al., 2020). Albeit, the presence of Nabs in a study group showed a sudden rise in the serum of deceased patients as compared to patients who recovered. This behavior was in congruence with the deranged state of immune system with high level of inflammation leading to the systemic breakdown during SARS-CoV infection (Zhang et al., 2006). The average number of days for the peak activity of Nab in recovered patients was 20 days whereas that in deceased patients it was only 14.7 days. The comparative analysis during the same number of days, showed that Nab titers were much higher in deceased patients than in those who recovered (Zhang et al., 2006). The heterogenous macrophage population plays a significant role at various stages of SARS-CoV infection in anti-S IgG treated lungs. The non-resolving pro-inflammatory macrophages with lowering levels of TGF-β presented conditions of severe lung injury, whereas increasing TGF-β expression had connection with resolving macrophages as evidenced in mild infections (Liu et al., 2019).

The condition of antibody-dependent enhancement (ADE) of viral infection has been reported where the complexes of antibodies and viruses, formed via Fc receptor-mediated endocytosis, establish infection in monocytes and macrophages (Olsen et al., 1992). In support of this observation with respect to SARS-CoV infection, an abatement in the production of pro-inflammatory cytokines resulted after FcγRs blockage (Liu et al., 2019). The RBD of the S protein also displays neutralization epitopes, prime host immune response and can be a potential vaccine candidate (Li, 2015). Other proteins of SARS-CoV like M and E also elicited antibody responses (Jin et al., 2005).

The Behavior of MERS-CoV

The role of HLA class II alleles have also been ascertained in cases of MERS-CoV infection (Hajeer et al., 2016). The S protein of MERS-CoV stimulates the expression of TLR negative regulators that impede viral clearance (Al-Qahtani et al., 2017; Mubarak et al., 2019). MERS-CoV infection studies also showed delayed but substantial response of all the three IFNs (type I, II, and III) (Menachery et al., 2014). Also, the infection witnessed an upsurge in pro-inflammatory cytokines and chemokines (IL-6, IL-1β, TNF-α, IFN-γ, and IL-8) (Lau et al., 2013). Here, higher levels of IL-8 were observed indicating the recruitment of neutrophils during MERS-CoV infection (Lau et al., 2013). The infection study also showed a detectable antiviral IFN-mediated response (Tynell et al., 2016). The MERS-CoV proteins antagonizing cellular targets are discussed in **Table 1B**. Data from a study on APCs, MDM (monocyte-derived macrophages) and MDDCs (monocyte-derived dendritic cells), which are residents of mucosal surfaces in the respiratory tract, showed that MERS-CoV readily established productive infection in human MDMs and immature MDDCs but failed to do so in the mature ones (Cong et al., 2018). Since immature MDDCs are efficient in uptake and processing of antigen whereas poor in stimulating T cells, this provides MERS-CoV with ample time for replication and thereafter dissemination for a successful productivity (Cong et al., 2018). Furthermore, clinical observations corroborated that the MERS-CoV showed systemic dissemination in patients as compared to SARS-CoV (Drosten et al., 2013). The chemoattractants, cytokines and chemokines were reported to be endurably produced during MERS-CoV infection that recruited immune cells in the lower respiratory tract leading to severe inflammation and hence tissue destruction. Lymphopenia and thrombocytopenia were also reported during MERS development (Zaki et al., 2012; Drosten et al., 2013). The role of CD8+ T cells and antibodies against the S protein of MERS-CoV, has been recognized as a protective candidate required for virus clearance in mice (Zhao et al., 2014). The RBD in the S1 domain of the S protein in MERS-CoV has also been shown to induce mucosal and systemic immune response (Li et al., 2019).

TABLE 1B | MERS-CoV proteins antagonizing cellular targets.

MERS-CoV	Proteins of the virus	Cellular targets	References
1.	Orf4a	Inhibition of IFN signaling	Yang et al., 2013
2.	Orf4b	Inhibition of IFN-I (NF-κB inhibition)	Matthews et al., 2014
3.	M, Orf5, Nsp3	Suppression of IFN-I	Yang et al., 2013, 2014
4.	Orf8b	Antagonizes IFN-β	Lee et al., 2019

Generally, dsRNA (formed after replication) acts as PAMP prototype in coronaviruses (Kindler et al., 2016). The entry of SARS-CoV has been shown to be restricted by IFN-induced transmembrane (IFITM) proteins (Huang et al., 2011). As studied in cell culture, IFN induction is diminished in both SARS-CoV and MERS-CoV infections (Kindler et al., 2016). SARS-CoV mouse model displayed pro-inflammatory monocyte-macrophage and cytokines that led to vascular leakage in lungs as a result of delayed induction of IFN despite the presence of higher levels of dsRNA (Kindler et al., 2016). This draws attention toward an important strategy for the virus in evading host antiviral response wherein both SARS-CoV and MERS-CoV stimulate the production of double-membrane vesicles (DMVs) acting as virus replication complexes that don't express any PAMPs (Kindler et al., 2016).

Mounting evidences show that the dysregulation of immune response and a surge in the production of chemokines and cytokines leads to a "cytokine storm" in the host, which has been delineated to be in congruence with the disease severity and poor prognosis, in both SARS-CoV as well as MERS-CoV (de Wit et al., 2016; Newton et al., 2016).

A "NO TRUCE" SITUATION BETWEEN THE TWO ARMS OF IMMUNITY

The condition of immunopathology arises when the invading pathogen awakens collateral damage due to the host immune response. The dynamism of host-virus relationship witnesses the attempts of a virus to eschew its visibility and the responses of the host through a myriad of challenges aiming at weakening the infection. The first line of defense, which combats the pathogen is innate immunity whereas reaction of adaptive response steps in after a few days. Infectious conditions resulting in an unleashed innate response with an absence of T cell supervision, leads to high damage in the host leading to death.

Studies on the comparative retrospection of SARS where patients who recovered against the ones who lost their lives, clearly support the fact that the innate arm of immunity cannot elicit a productive adaptive response, leading to succumbing of patients due to debilitating inflammation. SARS-CoV patients showed early expression of IFN-α, IFN-γ, CXCL10, and ISG-encoded proteins whereas only those who recovered exhibited adaptive immune response. Patients, who succumbed to the infection, had higher levels of CXCL10, CCL2, and ISG-encoded proteins with low levels of antibodies against the spike protein. It has been shown before with other viral diseases that the condition arising due to compromised gene regulation of HLA and antibodies leads to an aberrance in antigen presentation and production of antibodies. The same has also been documented during SARS-CoV infection (Cameron et al., 2007).

The Behavior of SARS-CoV-2

The main phenotypic expression of SARS-CoV-2 is ARDS, a condition coordinated by cytokine release syndrome (CRS), that bears a high degree of similarity with SARS-CoV and MERS-CoV (de Wit et al., 2016). The hypercytokinemia, reported in SARS-CoV-2 has been saliently associated with upregulated expression of chemokines and their receptors. The chemokines ranking ahead are CXCL17 and CXCL8, which are known to be recruiting neutrophils into the lungs (Zhou Z. et al., 2020). The studies based on the pathophysiology of SARS-CoV and MERS-CoV have proposed targets for therapeutic interventions aiming at the initial stage of the SARS-CoV-2 infection, but the actual challenge faced is the extreme inflammation arising at latter stage of the disease where ARDS escalates mortality rate (de Wit et al., 2016). As endorsed in the cases of SARS-CoV and MERS-CoV infections, the infliction of conditions like pneumonia and "cytokine storm" has been again known to be the underlying cause of cellular destruction in the host of SARS-CoV-2 (Chen G. et al., 2020; Chen N. et al., 2020). The testimony supporting the devastations caused during SARS-CoV-2 infection because of the immunopathogenesis can be well-witnessed in a number of reports (Channappanavar and Perlman, 2017; Chen G. et al., 2020; Chen N. et al., 2020; Chua et al., 2020; Giamarellos-Bourboulis et al., 2020; Guan et al., 2020; Huang et al., 2020). The clinical condition of SARS-CoV-2 infection is driven by dysfunctional immune system where profound lymphopenia, sepsis due to macrophage-activation and stumpy expression of HLA-DR on CD14-monocytes accompanied by excessive accumulation of IL-6 and IL1RA are observed (Giamarellos-Bourboulis et al., 2020). The presence of monocyte-associated chemokines (CCL2 and CCL8), appearance of circulating neutrophils and delayed expression of IFN-1, all are suggestive of the response to SARS-CoV-2 infection in the new human host (Bianco Mello et al., 2020). Going against the characteristic feature of SARS-CoV, the SARS-CoV-2 induces a robust IFN response, which is attributed to be the contributing factor for not only a higher percentage of milder or asymptomatic cases but also lower mortality rate (Zhou Z. et al., 2020). In severe cases, IFN-I expression could be at times be non-redundant or morbific, especially in epithelium tissues (Channappanavar et al., 2016; Ziegler et al., 2020). In agreement with this observation, cytokine treatment resulted in IFN-α driven expression of ACE-2, which could facilitate the enhancement of SARS-CoV-2 infection (Ziegler et al., 2020). The lung injury imposed during SARS-CoV-2 infection with reports of increased neutrophils and lowering lymphocyte counts clearly indicate about a possible interplay between virus PAMPs and host PRRs (Prompetchara et al., 2020; Tay et al., 2020). The innate arm of immunity mediated by RLRs gets activated by RNA 5'-triphosphate, hence addition of modified residues on 5'-triphosphate group abrogates RIG-1 activation. The NSP16/NSP10 heterodimer of SARS-CoV-2 has been reported to perform 2'-O methylation of the first nucleotide of its mRNA (Viswanathan et al., 2020). This feature would not only be helping in efficient and higher rate of viral mRNA translation but also evading recognition by PRRs. Similarly, the stress granule proteins (SGs) are known to provide scaffold for RLRs and virus mRNAs that stimulate the IFN pathway (Nakagawa et al., 2018). The N protein of SARS-CoV-2 has been shown to interact with SGs that disrupts the latter thereby affecting IFN signaling (Gordon et al., 2020).

There is a notable reduction in the circulating lymphocytes and CD4+ as well as CD8+ T cells, specifically in severe cases of SARS-CoV-2 infection (Chen G. et al., 2020; Peng et al., 2020). However, the hyperactivation status of CD4+ and CD8+ T cells showed high HLA-DR on the latter. The levels of differentially expressed IFN-γ and TNF-α in the CD4+T cells dropped down in severe cases as compared to milder infections of SARS-CoV-2. At the same time, perforins and granzyme B in CD8+T cells rose to higher levels in severe cases than those in milder cases. These observations have been implicated in the damage caused by SARS-CoV-2 resulting in the lowering of antiviral immunity in the host (Zheng H. Y. et al., 2020).

The Th1 cells have been shown to get activated resulting in the generation of granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN-γ, IL-1β, IP-10, and MCP-1 (Huang et al., 2020). On the contrary to SARS-CoV, SARS-CoV-2 infection showed elevated levels of IL-4 and IL-10 cytokines from Th2 cells (Huang et al., 2020). The presence of macrophage inflammatory protein 1α (MIP1A) and TNF-α are also significant in SARS-CoV-2 infection (Zhang Y. et al., 2020). The cytotoxic granules harbored in CD8+ T cells were high (Xu et al., 2020). Severe cases have been shown to have higher cytotoxic follicular helper (T_{FH}) cells as well as CD4+-CTLs in comparison to milder cases (Meckiff et al., 2020). The sera of convalescent COVID-19 patients show strong T cell response (Neidleman et al., 2020).

Lymphopenia is seen commonly and has been held as a critical factor in disease severity (Weiskopf et al., 2020; Yu et al., 2020). Patients with severe pulmonary inflammation had higher levels of virus induced expression of NKG2A/CD94 (NK group 2-member A, an NK inhibitory receptor) leading to the exhaustion of NK cells and CTLs (Zheng M. et al., 2020).

The patients of SARS-CoV-2 showed escalated viral titres in the first week that showed gradual decline over the second week. The overt symptoms accompanied with ascension of IgG and IgM antibodies against N and S (RBD) protein of SARS-CoV-2, was evident around the 10th day of illness (To et al., 2020; Wang et al., 2020). The high viral titers have been implicated in several practical outcomes during the early phase: infectivity potential of the patient becomes high (high transmissibility) and the virus gains an advantage of evading antiviral defenses within the host (Chen and Li, 2020). As observed in SARS-CoV cases, despite showing neutralizing antibodies in the serum, a small fraction of patients faced persistent inflammation eventually succumbing to the infection. This phenomenon was explained by ADE during virus infection (Fu et al., 2020). The same observation can be extrapolated in SARS-CoV-2 severe cases where patients suffer damaging inflammation even though neutralizing antibodies are secreted (Okba et al., 2020). Potent antibodies against S protein that bind specifically to RBD interface, was also shown to impede interaction of SARS-CoV-2 with ACE2 (Wang et al., 2020).

Table 2 represents various cellular targets of SARS-CoV, MERS-CoV, and SARS-CoV-2.

SARS-CoV-2: UNLEASHING THE TREND OF AGE BIAS

When the SARS epidemic emerged, the overall rate of mortality escalated on account of the lowering survival rate (50%) in patients above the age of 65 years. On the contrary, a marked disparity was seen in young patients (below 24 years) where survival rate was 100% (Peiris et al., 2004). Similar case was observed in MERS, where 45.2% mortality rate was observed in patients above the age of 60 years otherwise it was 20% for those under 60 years of age (Ahmed, 2017). In both the outbreaks of SARS-CoV and MERS-CoV, the existence of comorbidity in these patients elevated the risk of fatality with age (Chan et al., 2003; Yang et al., 2017). In the current pandemic of SARS-CoV-2, the susceptibility of patients with comorbidities lead to their poorer clinical outcomes (Guan et al., 2020; Mao et al., 2020).

ACE2 being one of the host membrane receptors of SARS-CoV-2, is known to be a negative regulator of angiotensin 2 (AngII), which raises blood pressure (Deshotels et al., 2014). The entry of SARS-CoV mediated by ACE2 has been shown to endocytose the receptor along with the virus after fusion, which results in diminished numbers of ACE2 receptors on the membrane leading to subsequent rise in serum levels of AngII (Kuba et al., 2005). The condition of ARDS has been well-known to cause acute lung damage where downregulation of ACE2 is in congruence with this model (Zhang and Baker, 2017). AngII has also been implicated in the immune cell differentiation and production of pro-inflammatory cytokines (Satou et al., 2018). The establishment of inflammation mediated via activation of NF-κB and IL-6 has been seen in the case of aged SARS-CoV patients (Brasier, 2010; Dediego et al., 2014). Since IL-6 has effect on cellular senescence, the rising levels of IL-6 in higher age cases of SARS-CoV-2 might correspond to their mortality (Hirano and Murakami, 2020). The expression of Th1 chemokines (CXCL9/10/11) and granzyme B have been shown to be at reduced levels in elderly patients of SARS-CoV-2 (Lieberman et al., 2020). The vaccine based preventive measures in aged population is an arduous task because of the poorer response in the older individuals (Katz et al., 2004; Kovacs et al., 2009). An investigation on the efficacy of SARS-CoV vaccine on aged mice also showed a waning effect (Deming et al., 2006).

As the pandemic has proceeded to establish the infection of SARS-CoV-2 in more and more individuals across the globe, there have been surprising reports wherein previously healthy young adults are showing severe COVID-19 infection. In a small cohort study, it was shown that lymphopenia could be a potential predictor of severe prognosis in younger adults with SARS-CoV-2 infection (Zhou C. et al., 2020). The reason for this observation still eludes us. Nevertheless, a few presumptions like overwhelming of the immune system by a sudden invasion of virus or individual specific immune response could explain this ambiguity where previously healthy immune systems are presenting ARDS. Another cross-sectional study reported younger patients with COVID-19 (median age 60 years) requiring intensive care unit (ICU) admission (Blake et al., 2020). It is surprising to note that the upper respiratory tract of both symptomatic as well as asymptomatic patients harbored similar viral loads (Zou et al., 2020).

The peripheral blood lymphocytes in children with SARS-CoV-2 infection are mostly within the range of normalcy, which is indicative of a controlled immune response (Cao et al., 2020).

However, one report from North American pediatric ICU has described COVID-19 infection severity in infants and children (∼80% had comorbidity) (Shekerdemian et al., 2020). Though the burden of the disease in children was lower in comparison to that of adults, this can't let us ascertain that SARS-CoV-2 spares this section of the population.

The SARS-CoV-2 seems to be breaking the rule of age bias that was seen in previous infections of SARS-CoV and MERS-CoV. The trend is of great concern because the generally younger population who tend to remain asymptomatic sources of transmission, risking the elder groups, could also fall prey to the severity of COVID-19 (Liao et al., 2020).

FUTURE PROSPECTS AND CONCLUSION

SARS-CoV-2 has gripped the globe with its relentless capacity for transmission. After the outbreaks of SARS-CoV and MERS-CoV in the past two decades, the emergence of a third coronavirus, SARS-CoV-2 has not only over-burdened our global health system but also shaken up our pride of conquering all aspects of life. In the case of SARS-CoV, the outbreak was impeded

TABLE 2 | Host cellular targets of various SARS-CoV, MERS-CoV and SARS-CoV-2 proteins.

Host target proteins	SARS-CoV proteins	MERS-CoV proteins	SARS-CoV-2 proteins	Stage of viral life cycle and function	References
ACE2	S		S	Attachment and entry Host cell receptor	Weiss and Leibowitz, 2011; Hoffmann et al., 2020; Lan et al., 2020; Valencia et al., 2020
DPP4		S		Attachment and entry Host cell receptor	Li, 2015; Seys et al., 2018; Valencia et al., 2020
IFITM	Known	Known	Not known	Entry restricted	Wrensch et al., 2014; Liao et al., 2019
Cathepsin L	S	S	S	Cleavage and activation	Kleine-Weber et al., 2018; Ou et al., 2020
Furin		S	S	Cleavage and activation	Coutard et al., 2020; Xia et al., 2020
TMPRSS2	S	S	S	S protein priming	Kleine-Weber et al., 2018; Iwata-Yoshikawa et al., 2019; Hoffmann et al., 2020; Meng et al., 2020
GSK3	N			Phosphorylation Facilitation of viral replication	Wu et al., 2009
IFN pathway			NSP13, NSP14,NSP15 ORF6 and ORF9b	Antagonize interferon pathway	Lei et al., 2020; Sa Ribero et al., 2020
NF-κB	N		NSP13, ORF9c	Inflammation	Liao et al., 2005; Dosch et al., 2009
E3 ubiquitin ligase: TRIM59 and MIB1			ORF3a and NSP9, respectively	Interference with antiviral innate immunity	Kondo et al., 2012; Gil et al., 2020
E3 ubiquitin ligase: RCHY1	NSP3	PLPro		Abolishing of p53 mediated antiviral activity	Ma-Lauer et al., 2016; Gordon et al., 2020
CAMK2D	NSP3			Interference in IFN pathway	Ma-Lauer et al., 2016
NUP98-RAE1			ORF6	Antagonize interferon pathway	Addetia et al., 2020; Gordon et al., 2020
Stress granule proteins: G3BP1 and G3BP2, LARP1, CK2, UPF1, MOV10			N	Abrogation of IFN signaling	Cascarina and Ross, 2020; Gordon et al., 2020
Stress granule proteins		p4a interacts with dsRNA		Inhibition of stress granule formation	Rabouw et al., 2016; Nakagawa et al., 2018
N-linked glycosylation enzymes	S and M	S	S	Facilitation of lectin-mediated virion attachment by S	Zhou et al., 2010; Watanabe et al., 2020; Zhao P. et al., 2020
Caveolin	ORF3a			Might regulate virus uptake and trafficking of viral structural proteins	Padhan et al., 2007
TRAF3 and ASC	ORF3a			Activation of NLRP3 inflammasome	Siu et al., 2019
RUNX1b	ORF3b			Immunomodulation	Varshney et al., 2012
KPNA2	ORF6			Modulation of host protein nuclear transport and IFN-1 signaling	Frieman et al., 2007
KPNA4 (importin-α3)		p4b		Evasion of innate response	Canton et al., 2018
Bcl-xL	ORF7a, E			Induction of apoptosis Lymphopenia	Tan et al., 2007
LFA-1	ORF7a			Attachment factor on leukocytes	Hänel and Willbold, 2007
Calcineurin/NFAT pathway	NSP1			Induction of IL2 Immunopathogenesis	Pfefferle et al., 2011
PHB1 and PHB2	NSP2			Might be altering cell cycle progression, cellular differentiation, mitochondrial biogenesis	Cornillez-Ty et al., 2009
dsRNA		p 4a		Sequestration of dsRNA, suppression of PKR-dependent translation, suppression of RIG-I and MDA5	Rabouw et al., 2016
Polyprotein-cleaving protease activity	PLPro (NSP3)	PLPro (NSP3)	PLPro (NSP3)	Deubiquitination Antagonize innate immunity	Grum-Tokars et al., 2008; Clementz et al., 2010; Fung and Liu, 2019
$PALS_1$	E			Breaching of alveolar wall	Teoh et al., 2010
Na^+/K^+ ATPase α-1 subunit and Stomatin	E			Reduction in activity of epithelial Na channel	Nieto-Torres et al., 2014; Schoeman and Fielding, 2019
hnRNPA1	N			Might regulate viral RNA synthesis	Shi and Lai, 2005
Cyclophilin A	N			Interferon pathway Might be crucial for virus infection	Yurchenko et al., 2010; Tanaka et al., 2013

ACE2, Angiotensin-converting enzyme; DPP4, Dipeptidyl-peptidase; IFITM, Interferon-induced transmembrane protein; GSK3, Glycogen synthase kinase; TRIM59, Tripartite motif-containing protein; MIB1, Mindbomb E3 ubiquitin protein ligase; NUP98, Nuclear pore complex protein; RAE1, Ribonucleic acid export; G3BP, Ras-GTPase-activating SH3 domain binding protein; mTOR-regulated translational repressor LARP1, La ribonucleoprotein; CK2, casein kinase; mRNA decay factors UPF1, Up-frameshift) and MOV10, Moloneyleukemia virus 10 like; NFAT, Nuclear factor of activated T-cells; IL2, Interleukin; PKR, Protein kinase R; PLPro, papain-like protease; Bcl-xL, B-cell lymphoma-extra large; $PALS_1$, Proteins associated with Lin seven; hnRNPA1, Heterogenous nuclear ribonucleoprotein; RUNX1b, Runt-related transcription factor; KPNA2, karyopherin alpha 2; LFA-1, lymphocyte function-associated antigen 1; PHB,),TRAF3, TNF receptor-associated factor3; ASC, apoptosis-associated speck-like protein containing a caspase recruitment domain; NLRP3, NOD-,LRR-and pyrin domain-containing protein),RCHY1, ring-finger and CHY zinc finger domain-containing 1; CAMK2D, calcium/calmodulin-dependent protein kinase II delta.

because the symptoms became evident before a patient became infectious. This made the containment of individuals easier. However, increasing lines of evidences have shown that most of the SARS-CoV-2 patients remain asymptomatic carriers. The data repertoire of studies conducted on SARS-CoV is germane to the explorations in the field of SARS-CoV-2 pathogenesis and treatment. The SARS-CoV antigens were reported to be present in organs like liver, pancreas, kidneys and cerebrum as well as bronchi, lungs and intestine (Matsuyama et al., 2005). The severe tissue damage coalescing with SARS, to some extent, has been suggested to be due to the proteases secreted in the target organs of SARS-CoV infected patients (Matsuyama et al., 2005). Apart from the aforementioned proteases, elastase secreted by neutrophils have been proposed to aid in the fusion of SARS-CoV envelope with the host membrane via cell-surface-mediated entrance. This has been subsequently known to enhance virus infection as compared to the canonical endosomal pathway of their entry (Matsuyama et al., 2005).

SARS-CoV and MERS-CoV emerged with a contagious advantage where they diminished the response of innate immunity in the host. One therapeutic approach here would be the use of agonists of the innate pathway that could reinstate antiviral state in the host. The proposal to use agonists as well as antagonists of TLRs has been a good choice that displays a broad-spectrum potential in therapeutics against some respiratory infections (Zhao et al., 2012; Shirey et al., 2013; Perez-Giron et al., 2014). In this line of evidence, data shows that the use of a hybrid IFN (IFN-α B/D) and controlled activation of TLR-3 by rintatolimod (Ampligen, poly I:poly C124), confers protection in cells/animals against immunopathology associated with cytokine storm (Barnard et al., 2006; Zhao et al., 2012; Perez-Giron et al., 2014). TLR-3 agonist, poly (I:C), has been shown to be protective against both SARS-CoV as well as MERS-CoV infection models (Zhao and Perlman, 2010; Zhao et al., 2014). Therefore, studies deciphering the role of PRRs in establishing antiviral state or identifying the points where virus antagonizes/escapes various PRR cascades, would deepen our knowledge on virus pathogenesis.

The gene polymorphism in the NFKBIA promoter (of gene NFKBIA, which codes for IκBα) has been shown to influence the innate arm of host immunity in various infections (Ali et al., 2013). A network-based analytical study on comparison between SARS-CoV and MERS-CoV infections strongly advocated a logarithmic scale upregulation in the gene expression of NFKBIA, which has been proposed to be a key regulator in the level of host immune response during virus infection (Moni and Lio, 2014). Therefore, studies on the genetic polymorphisms of NFKBIA in the current situation of SARS-CoV-2 infection could also help in understanding their impact on innate immunity.

Interestingly, SARS-CoV was shown to possess a single ORF8 during its earlier stage of spread, while the middle and late phases presented isolates with two fall outs of ORF8, i.e., 8a and 8b due to a 29-nucleotide deletion (Oostra et al., 2007). SARS-CoV ORF8b is known to activate NLRP3 inflammasome in macrophages accompanying the release of IL-1β (Shi et al., 2019). Hence, the presence of only ORF8b in SARS-CoV-2 can be focused in understanding the cause behind the ardent inflammation during infection.

The occurrence of various strains of SARS-CoV as well as MERS-CoV on account of the genetic diversity has raised concerns over the dimension and efficiency of potential vaccines (Consortium, 2004; Yang et al., 2005). The RNA viruses display higher rates of mutation, that selectively increases virus virulence, helps in escaping host defense and alters their tissue tropism (Consortium, 2004). A positive selection was seen in the case of SARS-CoV ORF 1a and S gene sequences, which showed most of the substitutions. Whereas, ORF1b was found to be the most conserved sequence of SARS-CoV (Consortium, 2004). In the same course of studies on the evolving nature of SARS-CoV, anti-S antibodies were also shown to bolster the entry of virus rather than neutralization (Yang et al., 2005). These opinions should be considered in further studies on the heterogenous infectious potential of SARS-CoV-2, which needs closer examination to understand the sequelae of infection.

A disturbed and exuberant innate immune response can cause devastating immunopathological condition in the host whereby our own immune cells, which are educated to fight against virus infections, cause immense destruction in the body tissues and organs. A perpetual evolution in the adaptive capabilities of the virus is highly dependent on the ever-changing environment and host behavior. Severity of a viral disease is in constant conflict with the need for the virus to be able to disseminate within the host. It evolves and adapts in its host. Hitherto, the virus provides its host with opportunities for clearance, but with the condition of keeping their transmission uninterrupted. As seen in the clinical course of SARS-CoV infection, three distinct disease phases were characterized. In the first phase, virus replication occurs robustly manifesting fever and cough, which subsides in a few days. The second phase, despite showing a progressive lowering in viral titers toward the end, exhibits high fever and hypoxemia resulting into pneumonia-like condition. The third phase sees the patients developing ARDS, often leading to death (∼20%) (Channappanavar and Perlman, 2017). As evident from the gradual decline in virus titer toward the very end of these clinical phases, the role of a completely dysregulated immune system resulting in a hyper state of inflammation in the host system.

Given the advancements in the field of research analytics and an ever-escalating data repertoire, the long-term sequelae of these coronavirus infections are still unforeseen. The increasing human globalization is now paying for the trajectory of progressiveness. Lastly, we will have to stop and think, whether we again want to become a target for the next pandemic virus.

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RM and JK conceived the idea. All authors contributed equally in generating the draft and final versions of the manuscript.

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miR-1207-5p Can Contribute to Dysregulation of Inflammatory Response in COVID-19 *via* Targeting SARS-CoV-2 RNA

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The present study focuses on the role of human miRNAs in SARS-CoV-2 infection. An extensive analysis of human miRNA binding sites on the viral genome led to the identification of miR-1207-5p as potential regulator of the viral Spike protein. It is known that exogenous RNA can compete for miRNA targets of endogenous mRNAs leading to their overexpression. Our results suggest that SARS-CoV-2 virus can act as an exogenous competing RNA, facilitating the over-expression of its endogenous targets. Transcriptomic analysis of human alveolar and bronchial epithelial cells confirmed that the CSF1 gene, a known target of miR-1207-5p, is over-expressed following SARS-CoV-2 infection. CSF1 enhances macrophage recruitment and activation and its overexpression may contribute to the acute inflammatory response observed in severe COVID-19. In summary, our results indicate that dysregulation of miR-1207-5p-target genes during SARS-CoV-2 infection may contribute to uncontrolled inflammation in most severe COVID-19 cases.

Keywords: microRNA regulatory network, SARS-CoV-2, macrophage recruitment, inflammatory response, competing RNAs, miRNA target prediction

INTRODUCTION

COVID-19 is the first worldwide pandemic in a globalized world. The short time since the outbreak is the reason why many aspects of the molecular interactions of SARS-CoV-2 in the human host are still unknown, especially its mechanisms at transcriptional level. The present study aims to unravel the role of human miRNAs in SARS-CoV-2 infection. miRNAs are short non-coding RNA molecules with a post-transcriptional regulatory function (Bartel, 2004). They bind complementary sequences in mRNA molecules, with the role of inhibiting the translation of their mRNA targets into proteins (Bartel, 2009). Host endogenous miRNA activity in viral propagation has been previously studied and many complex virus-specific mechanisms have been identified, although the precise role of miRNAs in viral infections is not yet fully understood (Bruscella et al., 2017). In this paper, we present the results of an extensive predictive analysis to identify human lung-specific miRNAs that may bind the SARS-CoV-2 RNA. Then, we

considered the already experimentally validated miRNA interactions with endogenous genes to identify the host's miRNA regulatory sub-network affected by SARS-CoV-2 infection, looking at the virus as a competing RNA (Sumazin et al., 2011). We finally evaluated the impact of such interactions on the expression profile of genes targeted by the identified miRNAs in human airway epithelial cells infected with SARS-CoV-2. Specifically, we identified miR-1207-5p as a possible regulator of the S protein in SARS-CoV-2 RNA. As so, we suggest that the viral RNA competes with the CSF1 mRNA, a known target of miR-1207-5p (Dang et al., 2016), leading to CSF1 overexpression. To support our hypothesis, several published transcriptional datasets were evaluated. The finding that the CSF1 gene is over-expressed in lung epithelial cells infected with SARS-CoV-2 supported our hypothesis. CSF1 controls the production, differentiation and function of macrophages and its overexpression may contribute to the acute inflammatory response observed in severe COVID-19.

METHODS

Transcriptomics Datasets and Analysis

Normal lung tissue expression profiles have been downloaded from TissueAtlas (Ludwig et al., 2016). Raw miRNA expression data from 18 lung control tissues were normalized with quantile normalization and the average expression level for each miRNA was computed. We used the average expression profile computed from all the 18 control tissues to identify the top 100 expressed miRNAs in normal lung tissue. **Table S1** summarizes the list of selected miRNAs and their average expression level in lung control tissues.

A wide collection of already available transcriptomics datasets with gene expression profiles after SARS-CoV-2 infection has been assembled from literature, as summarized in **Table S2**. When available, we considered the differential expression analysis results obtained by the authors. Otherwise, we preprocessed and analyzed the gene expression profiles as specified in column "Data analysis" of **Table S2**. When raw count RNAseq data was available, we used the DESeq2 (Love et al., 2014) R pipeline to compare infected *vs.* not infected samples, and the Benjamini-Hochberg procedure (Benjamini, 1995) to compute adjusted p-values. The univariate threshold of statistical significance was set at 5%.

SARS-CoV-2 Sequences

The RefSeq sequence NC_045512 was used as reference to predict the binding sites of human miRNAs on the viral RNA. A total of 15881 worldwide viral complete genomes was downloaded—updated to September 7th, 2020—from the Severe acute respiratory syndrome coronavirus 2 data hub of NCBI Virus database, by filtering for taxid = "2697049" and Nucleotide Completeness = "complete". Stability of particular viral genome regions was assessed by searching the exact match of the region in all the viral available genomes. To assess the statistical significance of the stability of each binding site, we associated a p-value with the number (m_{bs}) of viral sequences that showed a mutation in the region of the binding site. Such a p-value was calculated as the frequency with which a number of mutations larger or equal to m_{bs} was observed in all of the other regions with the same length of the binding site in the involved mRNA.

miRNA Target Prediction

Mature miRNA sequences were downloaded from miRbase, version 22. We used four miRNA target prediction tools to assess whether an RNA sequence is predicted to be a target of a miRNA: miRanda (Enright et al., 2003), PITA (Kertesz et al., 2007), Targetscan (Agarwal et al., 2015), and ComiR (Coronnello et al., 2012; Coronnello and Benos, 2013; Bertolazzi et al., 2020). miRanda script was used with -score 0 and -energy 0 settings. PITA and Targetscan scripts were used with default settings. ComiR was used to compute the ComiR score associated with the targets of each single miRNAs. For each miRNA we identified as highly predicted targets the genes that passed all the following conditions:

- miRanda binding energy, lower than -20;
- PITA $\Delta\Delta E$, lower than -15
- TargetScan Binding Site, 8mer or 7mer
- ComiR score, greater than 0.85

We used the localization of the binding sites predicted by PITA, miRanda and Targetscan to further restrict the set of targets by considering only the binding sites predicted by all the three algorithms. The resulting targets are named as highly predicted targets.

Experimentally validated miRNA targets were downloaded from miRTarBase, where only the validation methods with strong evidence (i.e. Reporter assays, RT-qPCR, and Western-blot based experiments) have been considered.

RESULTS

Five Human Lung-Specific miRNAs are Predicted to Target SARS-CoV-2 Viral Genome

Aiming to unravel the role of endogenous miRNA expressed in the human lung with respect to SARS-CoV-2 virus, we focused our analysis on the 100 most expressed miRNAs in normal lung (Ludwig et al., 2016), identified as described in *Methods*. We identified potential targets of these 100 miRNAs on SARS-CoV-2 RNA sequence (NCBI reference viral sequence NC_045512), using four miRNA target prediction tools (Enright et al., 2003; Kertesz et al., 2007; Coronnello and Benos, 2013; Agarwal et al., 2015) (see Methods). Only 15 miRNAs were predicted to target the viral RNA by all the four algorithms (**Figure 1A**). Among the predicted miRNA:viral RNA interacting pairs (specific target locations), six were identified by all four algorithms (**Figures 1B, C**).

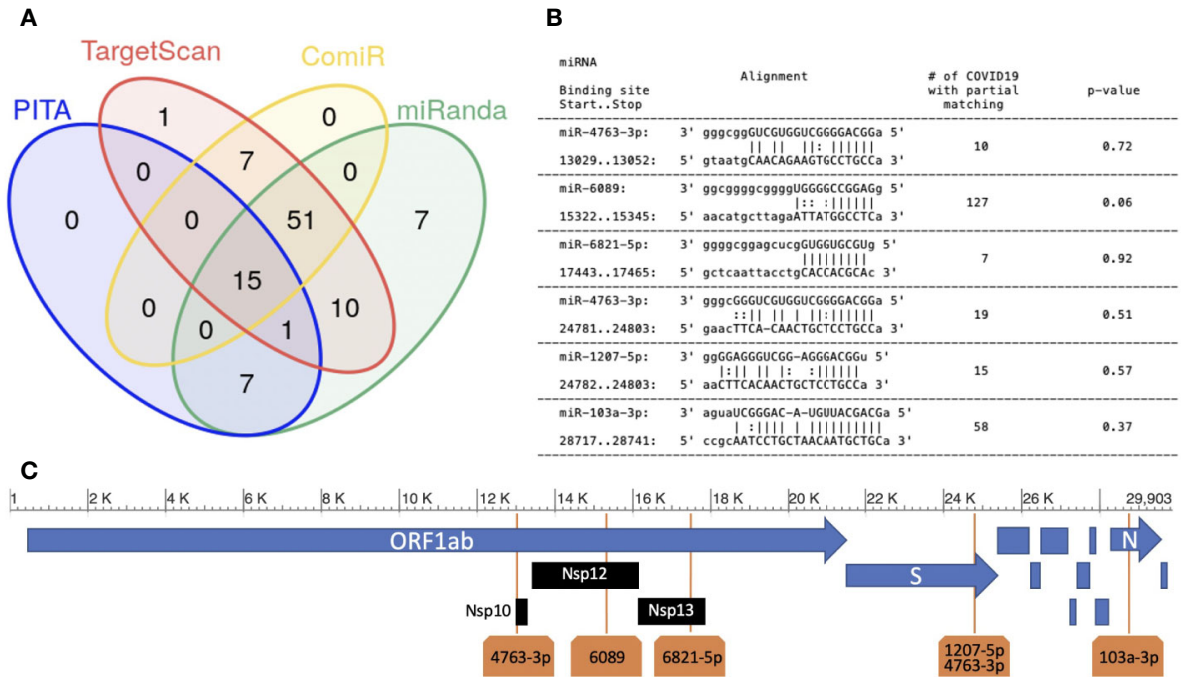

FIGURE 1 | Human miRNAs targeting SARS-CoV-2 sequence. **(A)** miRNA target prediction results of the 100 most highly expressed miRNAs in normal lung on SARS-CoV-2 viral RNA (NCBI Reference sequence NC_045512.2). Each group in the Venn diagram represents the number of miRNAs with target(s) on the SARS-CoV-2 sequence (algorithms used: PITA, Targetscan, miRanda and ComiR); **(B)** six miRNA:viral-RNA targets predicted by all methods ("high confidence targets"). Column-1: miRNA name, start/stop bases in the NC_045512 sequence; column-2: base alignment; column-3: number of SARS-CoV-2 sequences not containing an exact match for the binding site region; column-4: p-value (see *Methods*); **(C)** the location of the five high confidence targets on the SARS-CoV-2 genome.

The six sites were targeted by 5 miRNAs: miR-6089, miR-6821-5p, miR-103a-3p, miR-4763-3p, and miR-1207-5p. miR-4763-3p and miR-1207-5p miRNAs belong to the same miRNA family, sharing the same seed sequence (ggcaggg). In our analysis, we predict that they have a common binding site in the viral sequence, located in the region coding for the Spike (S) glycoprotein. Spike is a structural protein that allows Sars-Cov-2 to enter host cells by interacting with membrane receptors (Masters, 2006). Human miRNAs miR-6089, miR-6821-5p, and miR-4763-3p have their binding sites in the ORF1ab gene, specifically hitting the regions coding for Nsp10, formerly known as growth-factor-like protein (GFL), Nsp12, an RNA-dependent RNA polymerase, and Nsp13_ZBD gene, a helicase (**Figure 1C**). The three mentioned non-structural proteins are crucial in coronavirus replication, being part of a complex of 16 non-structural proteins entailed for viral RNA replication and transcription (Masters, 2006; Bouvet et al., 2014). miR-103a-3p binding site is located in the Nucleocapsid (N) protein coding region. N proteins are structural proteins, that play key roles during the packaging of the viral RNA genome (Masters, 2006). Whether the enhancement of the host's miRNAs regulatory machinery could inhibit the replication process or the production of the structural viral proteins, and as a consequence the virus diffusion through the host, is a hypothesis that needs to be experimentally validated and requires further investigation.

Stability of Predicted miRNA Binding Sites on SARS-CoV-2 RNA

The worldwide spread of COVID-19 infection exposes the viral genome to a high risk of mutation. For this reason, we checked the binding sites' sequence stability across the 15881 SARS-CoV-2 genomes annotated from all over the world in the NCBI virus database. To analyze such a stability, for each one of the six selected binding sites, we counted the number of viral sequences that presented a mutation. Results are reported in the third column of **Figure 1B**. We found that the binding regions are highly stable, which implies the consequent stability of binding-site predictions across the currently circulating viruses. In addition, we compared the occurrences of mutations in each binding site with the occurrences in any other region of the same length in the involved viral coding RNA, as described in the Methods section. The obtained p-values (see **Figure 1B**) indicate that the stability of all the six binding sites does not show a significant deviation from the one of the whole mRNA in which they are located, respectively.

Host mRNAs Competing With SARS-CoV-2 RNA are Overexpressed in Lung Epithelial Cells

The viral sequence, once expressed, can interact with the host's miRNA regulatory machine by sequestering the selected

miRNAs. Therefore, viral RNA may act as a miRNA sponge, with the same mechanism of competing endogenous RNA (Sumazin et al., 2011). Among the five selected miRNAs, two have been previously studied in detail. miR-103a-3p activity has been widely studied in different tissues, i.e. gastric and colorectal cancer or liver (Liao and Lönnerdal, 2010; Martello et al., 2010; Annibali et al., 2012; Chen et al., 2012; Yu et al., 2012; Zhang et al., 2012; Geng et al., 2014; Liang et al., 2015; Zhang et al., 2015; Asiaee et al., 2019), and a number of its targets has been validated. miR-1207-5p expression is high in the cytoplasmic fraction of human normal lung tissue while being reduced in cancer (Dang et al., 2016). miR-1207-5p has been first characterized as negative regulator of epithelial-to-mesenchymal transition (EMT) as it inhibits the expression of a number of genes involved in this process, including Snail, Smad2, Smad3 and Vimentin (Dang et al., 2016; Qin et al., 2016). In addition to its role in EMT, miR-1207-5p plays an important role in shaping the inflammatory milieu. In this respect, CSF1 (colony-stimulating factor 1, also known as macrophage colony-stimulating factor, M-CSF) has been reported as one of its direct targets (Dang et al., 2016).

In order to test whether infection of lung epithelial cells with SARS-CoV-2 cell infection affects the gene expression levels of the endogenous miRNA target genes, we used a recent dataset of gene expression profiles of human lung-derived cells infected with SARS-CoV-2 (Blanco-Melo et al., 2020). Authors examined the behavior of wild type adenocarcinomic human alveolar basal epithelial (A549) and airway epithelial (Calu3) cell lines. A549 cells show a low expression of ACE2 receptor, hence a limited coronavirus infection rate. Thus, the authors also analyzed A549 cells transfected with a vector expressing ACE2 (A549+ACE2). We used this dataset to analyze the transcriptional profiling of the experimentally validated targets of miR-1207-5p and miR-103a-3p.

Figure 2A presents the effect of viral infection on miR-1207-5p and miR-103a-3p endogenous target gene expression. Log-

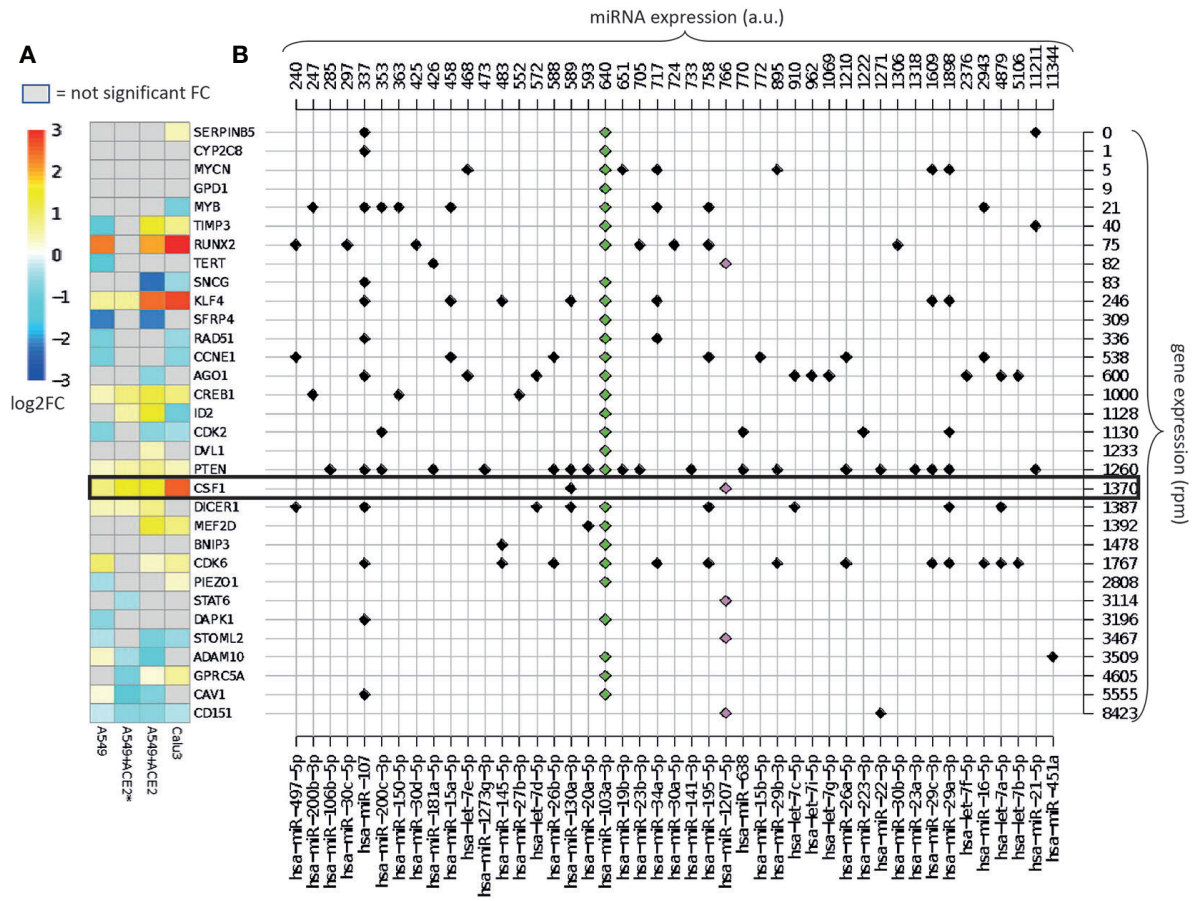

FIGURE 2 | Overview of the validated targets of hsa-miR-1207-5p and hsa-miR-103a-3p. **(A)** Heatmap of the log2FC in gene expression between SARS-CoV-2 infection *vs.* mock treatment in cells with different multiplicities of infection (MOI). Cells: A549 (low ACE2 expression), A549+ACE2* (ACE2-expressing A549 cells, low MOI = 0.2), A549+ACE2 (ACE2-expressing A549 cells, MOI = 2–5), Calu3 cells (MOI = 2–5) (GEO dataset GSE147507). Only the log2FC that are associated with adjusted p-value <0.05 are displayed. Target genes are ordered according to their average expression level in A549 cells. **(B)** Map of annotated interactions among the targets of hsa-miR-1207-5p (pink) and hsa-miR-103a-3p (green) and other highly expressed miRNAs in normal lung tissue miRNAs. miRNAs are ordered according to their expression level, shown on the top of the grid. Genes are in the same order as in panel **(A)** and their expression levels are shown on the right of the grid.

fold change (log2FC) values are calculated by comparing SARS-CoV-2 infected *vs.* mock treated cell lines (details in Methods). We expect that endogenous direct targets will increase their expression level following SARS-CoV-2 infection since viral RNA will compete with the endogenous RNA. Some of the analyzed targets behave as expected, especially the ones that are in the range of 1,000–2,000 reads per million (rpm), including CREB1, CSF1, PTEN, and DICER1. Consistent with the known A549 limited infection rate, the expression of these genes is enhanced in ACE2-expressing A549 cells, and even more in Calu-3 cells that are highly permissive to SARS-CoV-2 replication. These findings support our hypothesis that the viral RNA may act as a competing RNA for a selection of host miRNAs leading to the increase of the expression level of their endogenous targets.

Highly expressed targets, for instance ADAM10, are not up-regulated as expected. This is probably due to the fact that these genes might be modulated by other highly expressed miRNAs not sequestered by the virus. Alternatively, the sponge effect that we are hypothesizing is not effective when the mRNA is highly expressed.

Figure 2B presents the complexity of the miRNA-target network known up to now. Here we map all the experimentally validated interactions among the list of direct targets of miR-1207-5p and miR-103a-3p, and 45 of the 100 most highly expressed miRNAs in healthy lungs, that show at least one interaction. For instance, we observe that ADAM10, one of the targets of miR-103a-3p, is also regulated by miR-451a, the most highly expressed miRNA in lung. The presence of this regulator might be the reason why the expression of ADAM10 is not affected by the presence of the virus.

Binding of miR-1207-5p to SARS-CoV-2 RNA May Lead to Over-Expression of EMT-Related Genes and CSF1

miR-1207-5p has been first characterized as negative regulator of EMT by controlling the expression of several genes including SMAD2, SMAD3, SMAD7, CLASP1, ZEB1, and SNAIL1 (Dang et al., 2016; Qin et al., 2016). EMT processes favor fibrotic events. Of interest, current data suggest that pulmonary fibrosis after COVID-19 recovery could be substantial (Cabrera-Benitez et al., 2014; Merad and Martin, 2020; Spagnolo et al., 2020). Therefore, we tested the hypothesis that SARS-CoV-2 infection in bronchial epithelial cells may have an impact on the expression of these genes by reducing the availability of miR-1207-5p. **Figure 3** shows the results of the differential expression analysis for the genes involved in EMT that have been reported to be regulated by miR-1207-5p. The increase in their expression levels appears evident when cells are infected with SARS-CoV-2 virus, therefore supporting our hypothesis.

We further expanded our analysis by evaluating the impact of SARS-CoV-2 infection on the expression of CSF1. As reported in **Figure 2**, CSF1 is one of the host gene targets most upregulated following viral infection. CSF1 is a predicted target of 3 out of 5 of the miRNAs targeting the virus sequence: miR-4763-3p, miR-1207-5p and miR-6089. It is also an experimentally validated

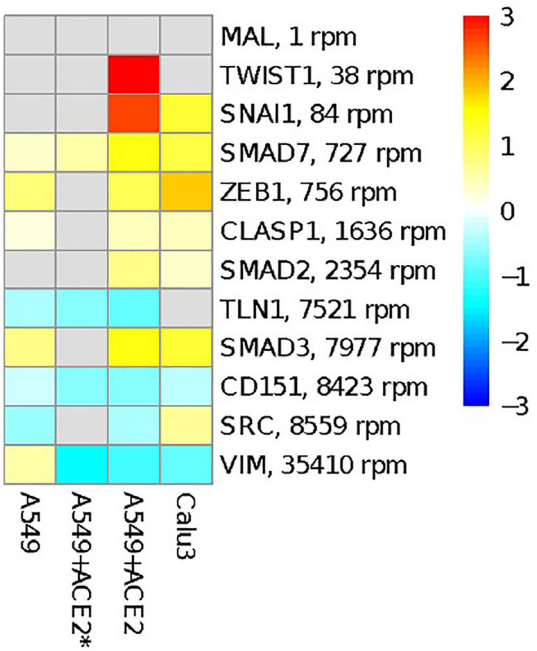

FIGURE 3 | Overview of genes involved in EMT process and reported to be regulated by miR-1207-5p. The heatmap shows the log2FC in gene expression between SARS-CoV-2 infection *vs.* mock treatment in the same cells as in **Figure 2**. Targets are ordered according to their average expression level in A549 cells.

target of miR-1207-5p (Dang et al., 2016). The only other known miRNA CSF1 regulator, among the 100 highly expressed miRNA in the lung, is miR-130a-3p, which is expressed at lower level than miR-1207-5p. CSF1 regulates the survival, proliferation, differentiation, and chemotaxis of tissue macrophages and dendritic cells (DC) that play a key role in innate immune responses. In the human lung, CSF1 can be released by airway epithelial cells in the airspace and its local concentration contributes to control the recruitment and activation of DC and macrophages (Louis et al., 2015; Moon et al., 2018; Turianová et al., 2019).

To further validate our hypothesis that the CSF1 mRNA is over-expressed after SARS-CoV-2 infection, we analyzed several recently published datasets as reported in **Table S2**. To this purpose, different types of experimental designs and platforms were taken into consideration. When available, we referred to the differential expression analysis performed by the authors. Specifically, we considered transcriptomics data analysis of infected *vs.* healthy samples from human lung biopsies as reported in (Vishnubalaji et al., 2020), bronchoalveolar lavage fluid (BALF) in (Zhou et al., 2020), peripheral blood mononuclear cells (PBMC) and BALF in (Xiong et al., 2020), and whole blood in (Ong et al., 2020). We also analyzed the single cell RNAseq data from whole blood reported in ref. (Wilk et al., 2020), infected NHBE cells in (Ravindra et al., 2020), and infected Calu3 cells in (Emanuel et al., 2020). Data sets obtained

by analyzing human samples were not useful to confirm our hypothesis. This can be due to several reasons. More specifically, the high-variability among patients, the cell heterogeneity of reported biological samples (such as bronchioalveolar lavage fluids and lung biopsies) with different efficiency of viral transfection and the low sample size make it really difficult to unravel fine regulatory mechanisms of virus-host interaction. On the contrary, when dataset derived from bronchial epithelial cells (both primary cells and cell lines) were analyzed, significant upregulation of CSF1 was observed therefore confirming our hypothesis. For example, Wyler et al. (Emanuel et al., 2020) performed gene expression profiles of SARS-CoV-2 infected Calu3 cell line. Overexpression of CSF1 in Sars-CoV-2 infected versus mock treated cells confirmed our hypothesis. Furthermore, in Ravindra et al. (2020) the authors performed single-cell RNA sequencing of human bronchial epithelial cells grown in air-liquid interface and infected with SARS-CoV-2. When looking at ciliated cells, the expression of CSF1 significantly increased in infected compared to mock cells. Of note, the expression of CSF1 was significantly higher in ciliated infected cells compared to bystander cells that remained uninfected in samples challenged with SARS-CoV-2. These findings suggest that viral replication inside the cells is required in order for CSF-1 to be over-expressed therefore supporting that a direct interaction between viral RNA and host miRNAs is required to alter the expression of CSF1 during infection.

DISCUSSION

In 10–20% of the cases, SARS-CoV-2 infections may progress to interstitial pneumonia and acute respiratory distress syndrome (ARDS) especially in patients with older age and comorbidities. Clinical features of severe COVID-19 as well as their systemic cytokine profile suggest the occurrence of macrophage activation syndrome (MAS) (McGonagle et al., 2020; Merad and Martin, 2020). High rates of viral replication have been listed among the factors that may drive severe lung pathology during infection by contributing to enhanced host cell cytolysis and production of inflammatory cytokines and chemokines by infected epithelial cells (Merad and Martin, 2020; Wen et al., 2020; Gardinassi et al., 2020). We propose that the high concentration of viral RNA in the cell may sequestrate miR-1207-5p therefore contributing to CSF1 release leading to enhanced macrophage recruitment and activation. In fact, increased release of CSF1 may represent a predisposing factor for MAS and cytokine storm secondary to viral infection (Akashi et al., 1994; Maruyama and Inokuma, 2010). Consistently, it has been recently reported that T-cell derived CSF-1, acting *via* intercellular crosstalk, may be associated with cytokine storm in COVID-19 (Wen et al., 2020). In our proposed model, infected bronchial epithelial cells may be a source of CSF-1 contributing to local and systemic inflammatory profiles. In addition, reduced availability of miR-1207-5p may also promote EMT events therefore favoring fibrosis (Cabrera-Benitez et al., 2014; Merad and Martin, 2020; Spagnolo et al., 2020). Although further experimental validation will be required to confirm direct interaction between miR-1207-5p and the SARS-CoV-2 genome, our proposed model has been confirmed using several published datasets. Results herein reported strongly suggest that upregulation of CSF1 due to interaction of miR-1207-5p with viral genome may occur when lung epithelial cells are infected with a high viral load. A limitation of the current study is the lack of data regarding protein levels and release. To address this issue, we carefully looked for published proteomics data in COVID19 literature, but, so far, no information about CSF1 protein levels has been published and therefore further studies will be carried out to address this point. Nevertheless, transcriptional and post-transcriptional control of mRNA levels represent a key regulatory step for most inflammatory mediators during infection. In this respect, the discovery of novel potential mechanisms that contribute to modulate the mRNA levels of a specific inflammatory mediator in the context of SARS-Cov-2 infection may represent a step forward toward a better understanding of virus-host interaction molecular mechanisms.

A wide analysis of the SARS-CoV-2 transcriptome (Kim et al., 2020) revealed the presence of several non-canonical sub-genomic RNAs. They consist in discontinuous transcriptions of the viral sequence, where the 5' leader region is fused to a non-conventional part of the genome. As a result, the obtained RNA contains only a portion of the viral mRNAs. It is tempting to speculate that they may play a role as competing RNA. Specifically, miR-1207-5p related binding site is located in the far downstream region of the viral gene Spike. As a consequence, almost all of the sub-genomic RNA sequences with the fusion occurring in the region of the Spike gene contain the miR-1207-5p binding site. Although, these sub-genomic RNA sequences do not have the coding potential to yield the S protein, they could still act as miRNA sponges.

To conclude, our results suggest that the miR-1207-5p family may interact with SARS-CoV-2 viral genome leading to deregulation of CSF-1, which may enhance inflammatory responses in COVID-19 patients, and promoting EMT, which can contribute to pulmonary fibrosis, a possible sequela of COVID-19. Further experimental validation will be conducted to confirm molecular mechanisms of host-virus interaction and to investigate their involvement in disease progression.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**. Further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

Conceptualization, MT, CCi, and CCo. Methodology, MT and CCo. Formal analysis, GB, MT, and CCo. Investigation, CCo. Writing—original draft, CCo. Writing—review and editing, GB,

PB, CCi, MT, and CCo. Supervision, MT and CCo. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.586592/full#supplementary-material

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REVIEW
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Fundamental Characteristics of Bat Interferon Systems

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Interferons are an essential component of the innate arm of the immune system and are arguably one of the most important lines of defence against viruses. The human IFN system and its functionality has already been largely characterized and studied in detail. However, the IFN systems of bats have only been marginally examined to date up until the recent developments of the Bat1k project which have now opened new opportunities in research by identifying six new bat genomes to possess novel genes that are likely associated with viral tolerance exhibited in bats. Interestingly, bats have been hypothesized to possess the ability to establish a host-virus relationship where despite being infected, they exhibit limited signs of disease and still retain the ability to transmit the disease into other susceptible hosts. Bats are one of the most abundant and widespread vertebrates on the planet and host many zoonotic viruses that are highly pathogenic to humans. Several genomics, immunological, and biological features are thought to underlie novel antiviral mechanisms of bats. This review aims to explore the bat IFN system and developments in its diverse IFN features, focusing mainly on the model species, the Australian black flying fox (*Pteropus alecto*), while also highlighting bat innate immunity as an exciting and fruitful area of research to understand their ability to control viral-mediated pathogenesis.

Keywords: bats, innate immunity, interferons, host-pathogen interaction, virus transmission

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AN INTRODUCTION TO THE INTERFERON SYSTEM

Interferons (IFNs) are a group of secreted cytokines that can induce an antiviral state of the host and primarily are responsible in inhibiting viral replication. They comprise part of the innate immune response and are one of the first and arguably most important lines of defence produced against viral infection, intended to limit the spread of a virus upon early infection (Weber et al., 2004). IFNs were first discovered in 1957 by Isaacs and Lindenmann (1987) who have identified interference of viral host antagonization in chick embryos. Since then, countless studies have been conducted in investigating the functional dynamics of IFNs in mammals. Three types of IFN exist in humans (type I, II, and III), which are categorized according to their amino acid sequences and their cognate receptor complex (Baker and Zhou, 2015). Type I IFNs consist of several genes including IFNα and IFNβ, which are both induced directly in response to viral infection, alongside IFNδ, IFNκ, IFNϵ, and IFNω, which all play less well-defined roles (Randall and Goodbourn, 2008). In the type II IFN group, there is only one single IFN called IFNϒ, which is secreted by T cells and natural killer (NK) cells of the immune system and is hence more associated with cell mediated immunity than innate

(Randall and Goodbourn, 2008). Type III IFNs have been discovered to consist of three main members; IFNλ1, IFNλ2, and IFNλ3 which are often referred to as interleukin (IL-)29, IL-28A, and IL-28B, respectively, in addition to the recent identification of IFNλ4, which is said to resemble IFNλ3 (Uze and Monneron, 2007). Both type I and type III IFNs are activated through the same signaling pathway and are secreted by viral-infected cells to elicit an antiviral state in infected and neighboring cells and work as part of the innate immune response (Onoguchi et al., 2007). IFNs interact with specific cellular receptors on cells, which activate signal transduction pathways that ultimately lead to the transcription of antiviral and immune modulatory genes, also referred to as IFN-stimulated genes (ISGs) (Le Page et al., 2000). A subset of ISGs can be directly induced by viral infection without the aid of IFNs and provide additional protection to infected cells.

INTERFERON INDUCTION

The IFN induction and signaling processes in response to a viral infection in humans have been discussed extensively elsewhere (Randall and Goodbourn, 2008). Briefly, IFN induction is stimulated following the recognition of pathogen-associated molecular patterns (PAMPS) which are molecular structures absent in uninfected cells, essential for the survival of the pathogen and distinguishable from "self" (Janeway, 1989). In RNA viral infections, PAMPS are features that are not usually present in cellular RNA, such as double-stranded RNA (dsRNA) or the presence of 5'triphosphate (5'ppp) and 5'diposphate (5'pp) groups (Killip et al., 2015). Pattern recognition receptors (PRRs) present in the host recognize these PAMPs and bind to them within infected cells. There are different classes of PRR that are involved in the activation of IFN pathways; the Toll-like receptor (TLR) family, RIG (retinoic acid inducible gene-) I-like helicase (RLH) receptors, nucleotide oligomerization domain-like receptors (NLRs) and cytosolic DNA sensors. The role of a certain PRR depends on the cell type and the nature of the viral stimuli; TLR and RLRs mainly respond to RNA viruses, whereas DNA sensors defend against DNA viruses. Upon recognition of PAMPs on the viral molecule, PRRs present at the cell surface or intracellularly in endosomes, signal to the host the presence of an infection. Intracellular signaling cascades are then activated which ultimately result in the expression of antiviral genes that orchestrate the early host innate response to infection (Mogensen, 2009).

TOLL/INTERLEUKIN-1 (IL-1) RECEPTOR-MEDIATED INTERFERON PRODUCTION

TLRs are the largest and most widely studied class of PRRs. They are glycoproteins that possess an extracellular domain containing leucine-rich repeats (LRRs) (which recognize a variety of ligands and bind to them), a transmembrane helix and a cytoplasmic signaling Toll/interleukin-1 (IL-1) receptor homology (TIR) domain (O'Neill and Bowie, 2007). TLRs are localized at the cellular or endosomal membranes such as the endoplasmic reticulum, lysosome or endosome where they recognize PAMPs via their LRR domain and transduce signals to the intracellular environment through the TIR domain. Antigen presenting cells such as macrophages and dendritic cells are arguably the most valuable cells that express TLRs; nevertheless, TLRs have been identified in most cell types (Iwasaki and Medzhitov, 2004). There are 10 different types of TLR identified in humans (TLR1-TLR10) which can be divided into subgroups dependent on the PAMPs that they recognize from different pathogens such viruses, bacteria, protozoa and fungi (Akira et al., 2006). TLRs signal via the recruitment of specific adaptor molecules such as MyD88 and TRIF (Kawasaki and Kawai, 2014) which lead to the activation of the transcription factors NF-κB, IRF3 and IRF7, ultimately leading to IFN and cytokine production. Three signaling pathways have been identified as essential in facilitating TLR-induced responses; those of mitogen-activated protein kinases (MAPK), NF-κB and IFN regulatory factors (IRFs). Despite NFκB and MAPK playing vital roles in the inflammatory response, IRFs are considered the most essential components required for IFN production. TLR1, TLR2, TLR4, TLR5 TLR6 and TLR10 located on the cell surface, recognize lipids and proteins and signal via the MyD88 pathway. Whereas TLR3, TLR7, TLR8, and TLR9 are intracellular, located at the endosome where many viruses un-coat their genomes and enter the cytoplasm. These intracellular TLRs recognize the nucleic acids; dsRNA, single-stranded RNA (ssRNA) and DNA, respectively and signal via the TRIF pathway (Majer et al., 2017). Upon engagement with ligands MyD88 activates MAPK and NF-κB which translocates to the nucleus, resulting in the synthesis of inflammatory cytokines such as TNFα. Alternatively, the TRIF pathway signals via the Toll-IL-1R domain-containing adaptor, inducing a signal cascade in which IRF3 or IRF7 translocates to the nucleus to induce the synthesis of type I IFNs; IFNα and IFNβ (Bagchi et al., 2007). TRIF is also able to activate through NF-κB leading to the production of inflammatory cytokines. TLR4 is unique in its ability to activate both the MyD88 and TRIF pathways (Hoebe et al., 2003).

CYTOSOLIC PRR SIGNALING

Although TLRs play a very significant role in sensing viral RNA at the cell membrane and in endosomes, it is apparent that additional sensing mechanisms must also take place inside of the cell, within the cytosol to aid in the contribution to host anti-viral defences.

Retinoic Acid-Inducible Gene-I Like Helicase Receptor-Mediated Interferon Production

RLH receptors are critical components of the anti-viral defence pathway. They are present in almost all cell types and consist of three RNA helicases; retinoic acid-inducible gene (RIG-I),

laboratory of genetics and physiology-2 (LGP-2) and melanoma differentiation associated gene (MDA5) (Thompson et al., 2011). RLHs recognize intracellular RNA that is introduced to the cell cytosol in a viral infection or is produced during viral replication. RIG-I senses 5'triphosphorylated uncapped ssRNA or short dsRNA and MDA5 recognizes long dsRNA. Upon recognition of this cytosolic viral RNA both RIG-I and MDA5 bind an adaptor protein called MAVS, which then initiates a signal transduction cascade. This in turn, leads to the activation of transcription factors such as NF-κB and IRFs causing IFN and inflammatory cytokine production. LGP2 has not been found to signal when interacting with viral RNA but seems to negatively regulate the other two RLHs in an unknown manner (Pippig et al., 2009). RIG-I and MDA5 consist of tandem N-terminal caspase activation and recruitment domains (CARD) in addition to a DExD/H box RNA helicase domain which has ATPase activity and a C-terminal repressor domain (CRD) (Thompson et al., 2011). Whereas LGP2 only consists of the RNA helicase domain as it lacks the two N-terminal CARD domains. RIG-I and MDA5 seem to recognize different classes of RNA viruses. As mentioned above, RIG-I is able to recognize viral RNA by interacting with 5' triphosphate "blunt ends" of RNA, whereas MDA5 PAMPs are unclear but apparently discriminate between self and non self RNA based on their sequence length (Thompson et al., 2011).

DNA Sensor-Mediated Interferon Production

TLR9 is primarily expressed in dendritic cells and B cells and is known to stimulate type I IFN production in response to foreign non-methylated CpG DNA in endosomes, via interacting with MyD88 to activate MAPK and NF-κB (Barber, 2011). There are many proposed cytosolic DNA sensors in addition to TLR9, including DAI/ZBP1, IFI16 and RNA Pol III. However, the two majorly characterized receptors consist of AIM2, a member of the PYHIN family, and cGAS (Xia et al., 2016). Upon stimulation in the cytosol by viral dsDNA, these sensors use the adaptor protein called stimulator of IFN genes (STING) to activate TBK1 and IRF3 and trigger type I IFN production. The signaling pathway linking DNA sensors to downstream effectors and TBK1 currently remains poorly characterized (Thompson et al., 2011). Other important pathways that are also activated by this method of intracellular DNA recognition include the inflammasome pathway, autophagy and cell death (Paludan and Bowie, 2013).

THE INTERFERON RESPONSE

Once activated, IFNs are secreted by infected cells where they enter the extracellular space and work to induce an antiviral state in the infected or neighboring cells in an autocrine or paracrine manner, respectively. In addition, they can also moderate innate immune responses to permit the action of antigen presentation and NK cells, but without the detrimental overactivation of pro-inflammatory pathways. Furthermore, IFNs can activate the adaptive immune system to induce specific T and B cell responses to the invading pathogen (Ivashkiv and Donlin, 2014). There are three types of IFN that are categorized based on which receptor they interact with; type I IFN interacts with receptors located in fibroblast cells, type II IFN receptors are located on endothelial cells and type III IFN receptors are largely found in immune cells. IFN receptors are comprised of heterodimers of two proteins with transmembrane domains, from which they recruit specific protein kinases that activate upon extracellular IFN binding to their cognate receptors (Sen, 2001). Activation involves dimerization of the IFN receptors which then activate downstream signaling pathways. Both type I and type III IFNs activate the JAK-STAT pathway. Type I and III IFNs both phosphorylate STAT1 and STAT2 and recruit IRF9 to form the ISGF3 complex which translocates to the nucleus and binds to the IFN-stimulated response element (ISRE) sequence. This induces the transcription of over 1,000 genes that act as antiviral defences, known as ISGs. Type II IFNs act differently to induce ISG transcription via the formation of a phosphorylated STAT1 homodimer, known as the gamma activation factor (GAF) complex, which translocates to the nucleus and binds the IFGAS sequence. The ISGs produced by all three IFN types act as antiviral effectors to protect host cells against pathogenesis via various methods, including; inhibition of translation, inhibition of viral entry, sequestration of viral mRNA translation, and the inhibition of viral transcription.

BATS AS VIRAL RESERVOIRS

Bats are grouped in the order Chiroptera and are one of the most abundant and geographically widespread vertebrates on Earth. The order Chiroptera can be subdivided into Yinpterochiroptera (consisting of megabats and some microbat species) and Yangochiroptera (composed of the remaining microbat families). Within these suborders high amounts of diversity are observed between bat size, ecological niches, diets, and morphology (Lei and Dong, 2016). There are over 1300 species of bats, representing more than 20% of all mammals on earth, deeming them the second most diverse mammalian group, after rodents (Wang and Anderson, 2019; Devaux et al., 2018). Bats are an important reservoir of zoonotic viruses and have been shown to harbor many viruses that are highly pathogenic to humans such as; rabies virus, Hendra virus and Nipah virus. They also harbor coronaviruses that are believed to have caused disease in humans after spillover events into intermediate hosts, including severe acute respiratory syndrome (SARS), Middle East respiratory syndrome (MERS), and the recently emerged 2019 novel coronavirus (CoVID-19) (Devaux et al., 2018). Similar genomes of these medically important viruses have been detected in bats where occasionally, only viral antibodies are identified in various bat species. As expected in reservoir hosts, despite harboring many viral species, bats rarely exhibit signs of disease. However, there are exceptions to this generalization as some viruses are known or suspected to kill bats including most, if not all, lyssavirus species, Tacaribe arenavirus, and Lloviu cuevavirus (Negredo et al., 2011; Cogswell-Hawkinson et al., 2012). Furthermore, the virus known as Zwiesel bat banyangvirus has

been recently discovered to kill bats from northern Germany (Kohl et al., 2020).

Bats are able to transmit viruses to humans in "spillover events" either directly or via an intermediate host. Significantly, the recent emergence of COVID-19, appears likely to have originated in bats and has entered human populations via an unknown intermediate host, likely traded in the Wuhan market at the epicentre of the outbreak. COVID-19 is the third identified highly pathogenic coronavirus to enter human populations (Zhou et al., 2020). Sequence comparison and evolutionary analysis of the SARS-CoV-2 genomes obtained from COVID-19 patients have revealed a high sequence similarity (~96%) with β-coronavirus of bats origin (Bat_CoV_RaTG13) (Zhou et al., 2020). This suggests that the bat and human COVID-19 share the same viral ancestor, although bats were not traded at the seafood marked in Wuhan (Wu et al., 2020). It is thought that certain stressors on bats such as disease or habitat loss for example, cause disruption of the viral-immune co-existence they possess. Upsetting this equilibrium therefore permits the multiplication of the virus, increasing its virulence and allowing transmission into other hosts (Rocha et al., 2020).

Bats possess unique characteristics that are distinguishable from other mammals which could possibly underlie their ability to harbor many viruses without showing clinical symptoms. However, it should also be understood that most other mammals are also able to harbor many viruses without exhibiting symptoms and it is the bats apparent unique immune characteristics that are important here in allowing them to harbor viruses. Bats are the only mammal capable of powered flight, allowing certain species to travel over large geographical distances during seasonal migrations and in pursuit of food where they may mix with other bat populations and hence contribute to the spread of viruses (Holland, 2007). Bats also have extremely long lifespans, Microchiroptera (microbats) for example, have life spans of around 25–35 years which is a longevity rarely seen in other mammals with similar body mass to metabolic rate ratios (Calisher et al., 2006) These features may potentially allow bats to host and spread viruses for longer durations and hence warrant future investigations. Moreover, the large population densities of bats and their mating behaviors, are extremely likely to increase the transmission of viral infections between individual bats and can henceforth also increase their transmission rate to other potential hosts.

These observations and studies highlight the need to explore diversity in the innate antiviral immune responses within this fascinating order of mammals and the seemingly unusual abilities of bats to control virus induced pathologies. In this section, bat IFN systems are discussed with the primary focus being the black flying fox (*Pteropus alecto*) and what we can learn from this bat as a model species.

THE BAT INTERFERON SYSTEM

Background

The black flying fox is most commonly used as the model species when studying bats and has therefore been used in most of the previous immune studies conducted on bats. Studies in the black flying fox and a few other bat species have identified many factors of antiviral immunity known in humans to be conserved in bats, including PRRs, IFNs, IFN receptors, and the ISGs they induce (De La Cruz-Rivera et al., 2018). Mutations in viral RNA species often result in the virus becoming biochemically "optimized" to exist in a particular host. It is therefore conceivable that RNA viruses have evolved alongside their bat hosts, allowing both to co-exist with each other. This could suggest why bats are able to harbor many different RNA viruses without exhibiting pathology. It is also suggested that bats may have adapted certain immune mechanisms that also aid in the establishment of a unique host-virus relationship (O'Shea et al., 2014), although the mechanisms underlying disease tolerance in bats remains largely unknown. One hypothesis proposed by (Baker and Zhou, 2015) suggests that bats are able to control viral replication early on in the immune response, via antiviral mechanisms and the stimulation of ISGs. There are two types of immunity shown in bats; innate and adaptive, innate immunity is the first line of defence against viruses and primes the adaptive response against the virus. This review is focusing largely on the IFN arm of the innate immune response, which are the first cytokines to respond to viral infection in bats.

Bat Pattern Recognition Receptors

As previously discussed, different PRRs have been identified in humans that appear to show certain homology to those found in bats. TLRs have been characterized from the black flying fox and the fruit bat Leschenault's rousette (*Rousettus leschenaultia*). Studies by Cowled et al. (2011a) have identified TLRs 1–10 in the black flying fox in addition to the nearly intact pseudogene TLR13 which is lacking in humans and most other mammals, but has only been previously identified in rodents (Zhang et al., 2013b). The TLR13 described in the black flying fox lacks a suitable start codon and contains 3 in-frame stop codons, suggesting that the pseudogene has recently been inactivated. Notably, the TLRs that are associated with nucleic acid sensing (TLRs 3, 6, 8, and 9), appear conserved between humans and bats, indicating the homology of bats viral recognition mechanisms with other mammals. Genomic analysis of TLR7 has indicated that it had evolved quicker in bats than other mammals; however, its function in bats still remains largely unknown. Baker and Zhou (2015) have suggested that the coevolution of viruses and bats may have caused changes in TLR7 that affect ssRNA recognition in bats.

Three types of RLH recognize viral RNA and DNA in the cell cytosol of most eukaryotic cells. Homologous to human RLHs; RIG-I, MDA5, and LGP2 have been identified in the black flying fox via transcriptome analysis (Cowled et al., 2011b). Bat RLHs show similarity to humans in their structure and expression (Baker and Zhou, 2015). Studies conducted by Cowled et al. (2011b) have proved that upon stimulation with synthetic dsRNA, all three helicases were upregulated in bat kidney cells, suggesting a functional homology in viral recognition between bats and other mammalian species.

DNA sensors identified in humans include AIM2 and IFIT16 which are associated with inflammasome assembly in addition to TLR9 and cGAS that are involved in IFN expression. There is

currently little known about bat DNA sensors; however, findings of a recent study showed that the most positively selected genes in bats are involved in innate immunity and the DNA damage pathway (Zhang et al., 2013a; Hawkins et al., 2019). NLRP3 is one of these genes, as identified in the black flying fox, to act as an inflammasome sensor via the activation of caspase-1, cleaving IL-1β, and IL-18. In humans, the inflammasome can also be activated by non-NLR proteins called AIM2 and IFIT16, belonging to the PYHIN family. However, these appear to be absent in all bat genomes sequenced to date, suggesting that bats have a lowered DNA-triggered inflammasome response to viruses (Ahn et al., 2016). In addition, TLR9 appears to be more highly expressed in bats than its other mammal counterparts. These discoveries could suggest that bats have evolved a unique IFN response in adaption to flight, which other mammals do not possess (Xie et al., 2018).

Bat Interferons: Production and Receptor Interactions

The IFN response is a key part of the innate immune system, acting as the first line of defence against viral infection. Type I and type III IFNs are induced in vertebrates in response to viral infection and are essential in establishing an antiviral state of host cells by the transcriptional activation of ISGs. Type I IFNs have been identified in five different bat species (Baker and Zhou, 2015) and are secreted from cells in response to viral infection, binding to IFN receptors to activate ISGs. The first transcriptome analysis of IFNs in any bat species was conducted by Zhou et al. (2016) in the black flying fox. They identified that the black flying fox consisted of only 10 type I IFNs, including three IFNα genes and thereby deduced that the black flying fox possesses a restricted type I IFN locus containing fewer IFN genes than other mammals. Interestingly, in contrast, IFNδ and IFNω genes appear expanded in the black flying fox, when compared to other mammals (Baker and Zhou, 2015). The contraction of the black flying fox IFNα locus together with its expanded IFNδ and IFNω genes, has not been observed in other species and could offer evidence for the unique host-virus symbiosis found in bats. It is feasible that the overexpression of IFNδ and IFNω compensates for the lack of IFNα response. In addition to the black flying fox, studies on other bat species have identified the IFNα locus to contrastingly appear expanded in the Egyptian fruit bat (*Rousettus aegyptiacus*), the greater flying fox (*Pteropus vampyrus*), and the little brown bat (*Myotis lucifugus*) collectively (**Table 1**) (Kepler et al., 2010; Pavlovich et al., 2018). Type III IFNs have recently been identified in bats and found to signal through the same pathway as type I IFNs but via a different receptor complex. Type III IFNs that have been identified in the black flying fox by Zhou et al. (2011a) include; IFNλ1 (IL-29) and IFNλ2 (IL28B), which appear to show homology to other mammals with similar loci and sequence length, indicating a functional conservation (Virtue et al., 2011).

Little is known about IFN production and the signaling pathways involved in bats as few studies have been conducted. Early studies by Stewart W.E II (1969) were the first to prove that IFN signaling pathways exist and are functional via the stimulation of bat cells with synthetic dsRNA (poly I: C) and lipopolysaccharide (LPS). Other studies that were conducted on different types of bat cells, appear to contradict each other. Splenocytes, which are immune cells, were taken from the black flying fox and experimentally infected with the bat paramyxovirus Tioman virus and resulted in the downregulation of type I IFNs, but also the upregulation of type III IFNs (Zhou et al., 2011b). These results could imply that this upregulation of type III IFNs could play a role in bats inimitable abilities to coexist with viruses. However, the roles of IFN-inhibitory proteins of these viruses were not investigated. In another experiment, infection of fibroblast cells from the black flying fox with henipavirus antagonized both type I and type III IFN production. The different results observed between the infected bat cells may be due to the immune specialty of splenocytes, giving them alternative IFN production mechanisms to fibroblast cells (Baker and Zhou, 2015). IFN production in bats generally appears similar to that of humans and other mammals. (Zhou et al., 2014) characterized all IRF family members from the black flying fox genome and found the IFNβ promotor region contains conventional IRF3 and IRF7 binding sites. IRF and NF-κB binding sites have also been identified in the promotor regions of IFNκ and IFNω in the serotine bat (*Eptesicus serotinus*) (He et al., 2014). IRF7 in humans is restricted to certain tissues, however an interesting finding by (Zhou et al., 2014) found that IRF7 mRNA in cells of

TABLE 1 | Nature of IFNs expression in currently studied bats species.

Bat Species	Type I IFN	Type III IFN	References
Australian Black Flying Fox *(Pteropus alecto)*	• Constitutively expressed IFNalpha • Contracted locus • No upregulation with Tioman virus • Antagonized by henipavirus	• Induced after viral infection with Tioman virus • Antagonized by henipavirus	Zhou et al., 2016 Zhou et al., 2011a
Egyptian Fruit Bat (Rousettus aegptiacus)	• No constitutive expression observed • Induced after viral infection • Extensive expansion of IFNw genes	Not determined	Pavlovich et al., 2018 Omatsu et al., 2008
Common vampire bat (Desmodus rotundus)	• Induction after polyI:C stimulation • Induction of selective IFN-stimulated genes	Not determined	Sarkis et al., 2018
Daubenton's bat *(Myotis daubentoniid)*	• Induction of IFN-stimulated genes • Induction of IFNs by virus and polyI:C	Not determined	Holzer et al., 2019

the black flying fox is more broadly distributed across tissues compared to mice and humans and is also constitutively expressed. This broad IRF7 expression may contribute to the ability to activate IFN responses in multiple tissues and cells and thereby respond more rapidly to infection. However, this has only been identified in a single bat species and hence requires further research to explore the IRF7 expression in other bats to determine whether it is a feature identifiable in all bat species.

The type III IFN receptor (IFNλR) has been well characterized in the black flying fox (Zhou et al., 2011b). It was revealed that IFNλR is transcribed in virus-infected bats, regardless of the suppression of type I IFNs. The IFNλR in the black flying fox is comprised of two genes; IFNλR1 and IL10R2 and appears homologous to the type III receptor found in humans and other mammals. IFNλR is widely distributed at the tissue and cellular level, present in both immune and epithelial cells, where it is receptive to IFNλ treatment and therefore presents as a functional receptor (Zhou et al., 2011b). This distribution is consistent with previous findings that suggest type III IFNs play a more vital role in bat antiviral immunity, as type III IFNs are upregulated, while type I IFNs are simultaneously downregulated upon viral invasion. Little is known about the type I IFN receptor in bats as no experimental studies have been carried out. However, genomic analysis has been conducted on certain bat species and found that the IFNAR1 gene has undergone positive selection in the vesper bat species *Myotis davidii*, but interestingly not in the black flying fox (Zhou et al., 2011b) and the consequences of this on their immune response is currently unknown.

Once type I and type III IFNs have bound to their cognate receptors, they activate the same signaling pathway, called the JAK-STAT pathway, which ultimately leads to the activation of antiviral effectors and ISGs, as detailed in **Figure 1**. Little work has been directed toward identifying IFN signaling in bats, however experiments by (Brzozka et al., 2006) found that stimulation of bat cells with human IFNα, resulted in the translocation of STAT1 into the nucleus, similar to the activation found in other mammal species. Therefore, it can be deduced that bat IFN signaling downstream of receptors appears to be comparable to other mammals.

IMMUNE FEATURES IN BATS

The black flying fox can harbor certain viruses without showing signs of disease but can still transmit the virus to other mammals and humans in spillover events, in which the virus can cause pathology in the infected host. It is therefore imperative that this

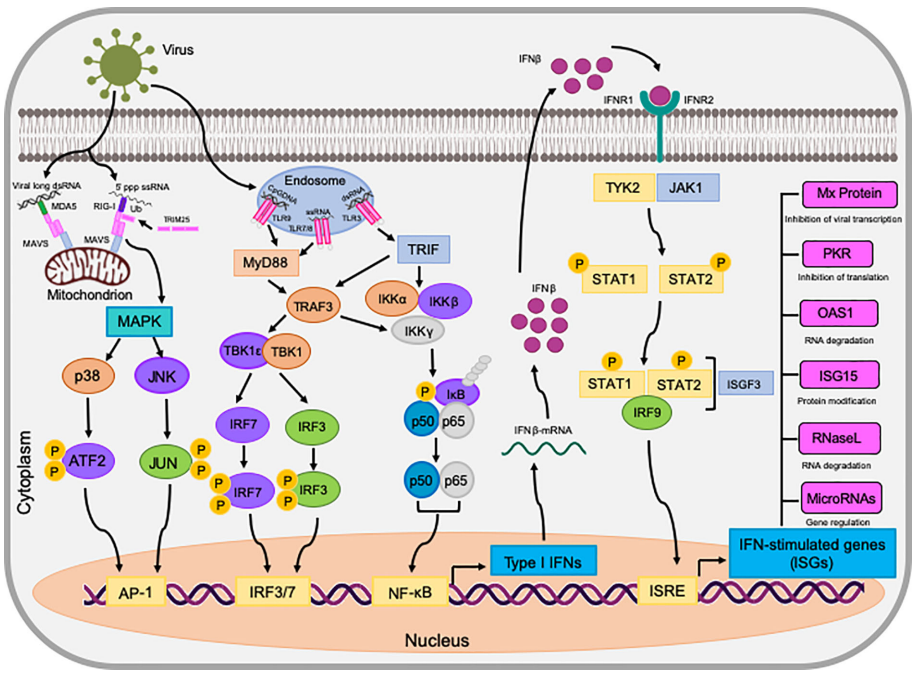

FIGURE 1 | The induction of IFNs and the antiviral state they exhibit in bat (*P. alecto*) cells. dsRNA is detected by either RIG-I/MDA5 or TLRs on endosomes, initiating downstream signaling via MAVS, TRIF, and TRAF3. These adaptors activate the transcription factors IRF3, IRF7, NF-κB, and AP-1 via the assembly of multi-protein complexes. Upon activation, these transcription factors translocate to the nucleus and stimulate the transcription of interferons, such as IFNβ. IFNβ then binds to its cognate receptor complex IFNAR via autocrine and paracrine manners to activate the JAK-STAT pathway. This terminates at the activated ISGF3 transcription factor which in turn, translocates to the nucleus and initiates the transcription of genes in ISRE promoters known as ISGs. ISGs then act in a multitude of ways to establish an antiviral state in the cell against invading pathogens.

ability as to how these bats remain unharmed while harboring these viruses is unearthed. It is suggested that the evolution of many characteristics of bats such as being the only flying mammal, having long life spans, their nocturnal abilities and reproductive mechanisms, may all contribute to the hypothesized uniqueness of their immune response that has not been observed in any other species. This section will discuss comprehensively the different plausible traits that bats possess, focussing largely on the black flying fox, that may prove advantageous in coexisting with viruses.

Unusual IFNα Expression in Bats

Research by (Zhou et al., 2016) was the first to characterize the type I IFN locus in the black flying fox and compare it to other species. They found that bats contain fewer IFN genes than any other known mammal and found that the black flying fox only possesses three IFNα genes. In addition, they revealed that IFNα genes are constitutively expressed in unstimulated bat cells and tissues where their level remains unaffected by viral infection. Infection of *P. alecto* kidney PaKiT03 cells with two bat-borne viruses (Hendra virus and Pulau virus) caused no change in the constitutive IFNα expression pattern. This expression has not been observed in any other species which suggests its significance in the bats ability to coexist with viruses. To ensure that the constitutive IFNα expression was unspecific to the black flying fox, they conducted the same studies using the lesser short nosed fruit bat (*Cynopterus brachyotis*) and found similar results of continually high IFNα expression across all tissues, regardless of viral stimulation (Zhou et al., 2016). Functionality of the black flying fox IFNα proteins were assessed using transfection experiments in human HEK293T cells, which displayed successful induction of ISGs in all three black flying fox IFNα proteins (Zhou et al., 2016). Findings suggest that IFNα is not upregulated in response to viral dsRNA sensing in these bats, but instead the high baseline levels of IFNα means that it can still be detected in the absence of immune stimulation. Contraction of type I IFNs in the black flying fox and the differences in their expression patterns, is consistent with the "less is more" theory that natural selection can produce mutations that favor fewer functional genes, but with advantageous consequences to the host (Olson, 1999). Additionally, Zhou et al. (2016) have determined that bats use fewer IFNα genes to perform functions, in comparison to IFNαs identified in other species, by using a system that is constitutively primed to respond to viral infection. Research by Shaw et al. (2017) provides further supporting evidence to the constitutive expression of IFNα as they identified the basal transcription level of type I interferome in both megachiropteran and microchiropteran cells to be significantly higher than the other species studied (**Table 1**). Despite possessing fewer IFNα genes, the ubiquitous and constant expression of IFNα in some bats may provide them with an effective system for controlling viral replication, allowing them to exist in a constantly active antiviral state. It can be concluded that the observable antiviral mechanisms in bats that differ between species can is likely to have arisen via convergent evolution and tolerance mechanisms identified in the black flying fox should not be generalized across all bat species. This highlights the need for further exploration of IFNα expression in further bat species. Furthermore. the contrasting expression patterns of IFNs already identified in some bat species highlights the inter-species diversity in bats when mounting immune responses against pathogens and eludes to the existence of additional features between different bat species.

IRF7 Distribution

Studies by (Zhou et al., 2014) have used the black flying fox as a model species to explore the role of the IFN system in the regulation of viral replication in bats. Bats appear to show a higher expression and wider distribution of type III IFN receptors than type I, suggesting that type III IFNs have a key role in the black flying fox's antiviral immunity (Zhou et al., 2011b). Significant evidence was provided by (Zhang et al., 2013b) that backed the positive selection of genes in the IFN pathway, including TLR7, TBK-1, IFN-ϒ, ISG15, and RIG-1 which may be due to the co-evolution of bats and viruses, and may assist in the ability of these bats to asymptomatically coexist with viruses. As IRF7 is a master regulator, central to the IFN-dependent immune response, Zhou et al. (2014) performed sequence and functional analysis of the black flying fox IRF7 to provide evidence for the conserved IRF7 functionality observed in bats, despite its sequence variation. IRF7 is expressed in low levels in most cell types in other animals but is highly expressed in immune cells such as dendritic cells and is induced in type I IFN mediated signaling in these cells via the activation of TLR7/9 and the MyD88 dependent signaling pathway (Ning et al., 2011). Results from Zhou et al. (2014) have confirmed that the activity of bat IRF7 is conserved but found it to have a wider tissue distribution and unique expression pattern in both immune and non-immune cells. It is hypothesized that the broad distribution of IRF7 may increase the ability to activate the IFN response in a wider range of tissues than found in other mammals, enhancing bats antiviral immunity. There is a lack of data on the tissue distribution of IRF7 in other bats and also in other mammals except from human, mice and horses; however, the broad distribution of IRF7 in bats has also been observed in at least five species of fish and was hence hypothesized to play a key role in fish antiviral immunity (Zhang et al., 2003). Zhou et al. (2014) suggest that further analysis of cell types responsible for the IRF7 expression in bats is required but suggested that the constitutively expressed IRF7 in a broad range of tissues may result in a faster and stronger IFN production upon viral infection (Ning et al., 2011). Upon analysis of the IRF7 protein sequence in the black flying fox, there is an apparent deletion at around 260 amino acids, when compared to its human counterpart. The deleted section lies between two domains; the constitutive activation domain (CAD) and the virus-activated domain (VAD). Evidence suggests that this could be an evolutionary deletion attributed to the ability of bats to co-exist with viruses, allowing the IRF7 to remain active but functionally different to its human counterpart.

However, similar to IFN expression diversity in the black flying fox and the Egyptian fruit bat, there may be species level disparity, and this thereby warrants future investigations.

Positive Selection of Bat IRF7 and Antiviral Responses

IRF3 and IRF7 are critical transcription factors in driving the expression of IFNs (Randall and Goodbourn, 2008). Sequence analysis of putative IRF3 sequences reveals evolutionary differences among bats when compared to other mammals. A recent functional study in the big brown bat (*Eptesicus fuscus*) indicated a mammalian-like MERS or dsRNA-induced stimulation of IFN production (Banerjee et al., 2019). In contrast, silencing of IRF3 in the big brown bat resulted in suppressed IFN activation with similar stimuli. However, the molecular mechanism of this induction remains elusive. Recently, computational analyses of bat IRF3 revealed a highly conserved serine residue at position 185 (S185) in 7 of the 11 examined bat species. Replacement of S185 with D185 in bat IRF3 conferred an enhanced protection against model vesicular stomatitis virus. Interestingly, substituting the leucine residue of human IRF3 with corresponding serine residue from bat IRF3 significantly enhanced antiviral protection in human cells (Banerjee et al., 2019; Banerjee et al., 2020). These insights support the notion that bats have acquired multiple adaptations in their antiviral immune responses to co-exist with pathogens. While these initial studies are projecting an interesting side to the bat's antiviral responses, it remains to be explored if this positive selection is species-specific and its biological relevance against emerging and bat-borne viruses.

Dampened Nucleotide Oligomerization Domain-Like Receptor Family Pyrin Domain Containing 3 Inflammasome Response

The NLR family pyrin domain containing 3 (NLRP3) inflammasome sensor has been proven central to age related and viral-induced inflammation in humans and other mammals.NLRP3 is vital to the inflammasome of cells and its role is to recognize cellular stresses such as mitochondrial damage or oxidative stress, in addition to bacterial or viral infections. NLRP3 is known to respond to a myriad of viruses, including bat-borne viruses such as rabies and influenza (Ahn et al., 2019). Until recently, nothing was known about NLRP3-mediated inflammation in bats, but it was hypothesized that despite the NLRP3 inflammasome existing as a central player in viral infection in bats, it differs between bats and other mammals. Leading research by Ahn et al. (2019) demonstrated an overall dampened activation of NLRP3 in bat primary immune cells, when compared to their human or murine counterparts (**Figure 2**). Bats were shown to display a dampened host inflammasome response to both viral and bacterial infections

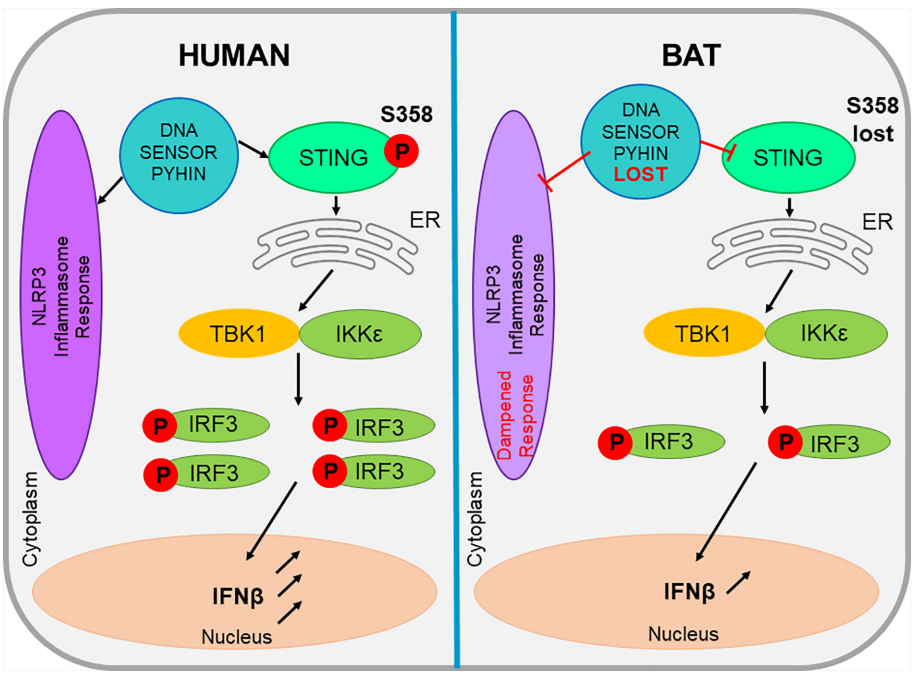

FIGURE 2 | The comparison of bat and human STING and inflammasome activities within a cell. The evolutionary loss a DNA sensor belonging to the PYHIN gene family leads to a dampened NLRP3-mediated inflammasome response. This loss also impacts the action of STING, which in bats lacks a serine residue, reducing its functionality. These factors all appear unique to bats and all contribute to the reduced level of IFN produced in bat cells.

and cellular danger signals. (Ahn et al., 2019) tested bat cells by infecting them with three different zoonotic RNA viruses and found that viral loads remained unaffected in the bat primary immune cells. This was due to the dampened transcriptional priming and decreased functional capacity of bat NLRP3. Upon testing wild and experimentally infected bats, it was evident that they were able to tolerate viral diseases, even with a high viral load present in the host. Evidence shows that bats have naturally dampened stress-related and pathogenic sensor induced responses which coincides with their ability to exist as asymptomatic viral reservoirs (Ahn et al., 2019). The dampened NLRP3 response that has been identified in bats, supports the theory that they possess an enhanced immune tolerance, as appose to an enhanced antiviral defence (Ahn et al., 2019). Therefore, this aids in the explanation of inflammasome significance in disease tolerance in bats, in contrast to the pathogenesis observed in spillover hosts.

Absence of Pyrin and HIN Domain Gene Family

In addition to the positively selected NLRP3 inflammasome sensor, the entire pyrin and HIN domain (PYHIN) containing gene family appears evolutionarily lost in bats, suggesting a dampened DNA-triggered inflammasome response as shown in **Figure 2**. The PYHIN gene family are important immune sensors of intracellular and foreign DNA and activate the immune response. They are the only DNA sensors capable of activating the inflammasome (Hartlova et al., 2015). Previous genome analysis of two bat species (*P. alecto* and *M. davidii*) revealed the absence of the PYHIN gene family in both species. Studies by Ahn et al. (2016) further these findings and analyze 10 different bat species, which covered four of the five major bat lineages and confirmed the complete loss of this gene in all species, despite the presence of the PYHIN locus. The only minor discrepancy in this study was the identification of a truncated AIM2 gene in the Parnell's moustached bat (*Pteronotus parnellii*,) which is thought to be where the bat PYRIN sequence clustered with AIM2, indicating the presence of a functional AIM2 gene in the bat common ancestor that was lost during evolution. All other major groups of placental mammals possess at least one gene member, whereas most bats appear to have lost the entire PYHIN gene family as a loss of function evolutionary event (Ahn et al., 2016). The unique absence of PYHIN genes in bats suggests it may be an important adaptation that is possibly induced by flight and affects DNA sensing and inflammasome activation (Ahn et al., 2016). Bats are the only mammals capable of flight, which is considered metabolically costly; however, bats have the ability to increase their metabolic rate up to 34 times their resting rate to compensate for this (Thomas and Suthers, 1972). The exclusive loss of PYHIN in bats suggests an important adaptation for flight. Despite still possessing other cytosolic DNA sensors, such as cGAS, STING, and ZBP1, PYHIN is the sole activator of the inflammasome (Dempsey and Bowie, 2015). Therefore, its deletion may enable some bats to limit excessive inflammation activation and in turn, regulate the type I IFN response which is normally triggered by PYHIN proteins recognizing the DNA damage and release of self-DNA from the metabolic activities of flight. There is a likelihood that the increased exposure of bats to many zoonotic RNA and DNA viruses when compared to other mammals that do not cover large distances, may be the evolutionary driver of PYHIN loss, or contrastingly, the loss of PYHIN may allow for this bat-viral co-evolution (Ahn et al., 2016). The loss of PYHIN in bats is also hypothesized as a basis for the long lifespans that bats exhibit.

Dampened Stimulator of Interferon Genes -Dependent Interferon Activation

Cytosolic DNA either produced from flight or viral infections imposes selective pressures on bat DNA sensors such as the PYHIN gene family mentioned previously. This results in a dampened sensing mechanism and downstream IFN production to avoid overreaction on a regular basis during flight or due to viral co-existence (Xie et al., 2018). While bats detect and respond to RNA viruses, studies have shown that the response of bats to DNA infections is dampened. Along with the absence of the PYHIN gene family, the ability of STING, an essential adaptor protein involved in multiple DNA-sensing pathways to induce IFN expression, also appears dampened in bat cells. This can be attributed to the mutation of the serine residue at position 358 (S358) in STING. Xie et al. (2018) carried out sequence and functional analysis to identify the dampened, but not diminished, response of STING in bats (**Figure 2**) against herpes simplex virus (HSV) replication. By experimentally reversing the mutation, it was revealed that STING functionality was restored in the bat cells (Xie et al., 2018). The mutation of the serine residue at the phosphorylation site 358 in bats, resulted in the impaired ability to activate downstream IFNs (Liu et al., 2015) and was identified in every known STING protein of bat. This STING dampening explains the reduced ability of bat cells to detect self-DNA and exogenous DNA and is speculated to be a side effect to the evolution of powered flight in bats. During flight, the body temperature of bats can reach over 41°C. The elevated body temperature and high metabolic rates in bats can produce reactive oxygen species (ROS) which in turn cause DNA damage and the release of cellular DNA into the cytoplasm. Therefore, it is theorized to overcome this, bats have undergone a positive selection for various genes involved with DNA repair, which itself causes consequences for antiviral responses. It is unlikely however, that bats have lost all DNA-sensing machinery, as DNA viruses have been identified and isolated from many bat species (Banerjee et al., 2020). Future studies are required to understand the adaptations bat cells have evolved to sense DNA viruses while also limiting the detection of self-DNA. Theoretically, the evolution of flight in bats may have caused consequences for the immune response in minimizing the DNA damage associated with high metabolic rates. Also, exogeneous DNA sensing pathways may have been dampened to reduce self-DNA mediated immunopathology which in turn may have consequences for viral DNA detection in bats. Further research is also required to determine if bats have evolved novel mechanisms to sense and respond to exogenous and self-DNA

(Ahn et al., 2016). STING remains conserved in every other mammal; therefore its dampened expression could possibly provide bats with an advantage that would likely be associated with flight and the ability to maintain an effective, but not over-reactive response to co-existing with some viruses (Xie et al., 2018).

Immunogenetics and Viral Reservoir Potential of Bats

Recently, developments from the Bat1k project which started back in 2017 have now published six near-complete annotated bat genomes. Through screening, they investigated gene loss, gain and selection to identify novel genes that are likely associated with viral tolerance within bats. These results will hence prove extremely useful in expanding existing knowledge of bat immunology within this fertile field of research (Jebb et al., 2020). Upon performing unbiased genome-wide screens for gene changes within the six bat species, Jebb et al. (2020) identified nine genes that have undergone positive selection in the bat ancestor. Among these are the genes LRP2 and SERPINB6 which have known roles in hearing. The project deduced that LRP2 has an amino acid substitution that is only found in bat species that utilize echolocation, indicating its significance in hearing. Pteropodid bats are described as exhibiting a different amino acid within LRP2 and these bats do not have laryngeal echolocation. These results suggest that if these gene mutations are related to echolocation, then this would prove the origin of echolocation in bats with evidence for echolocation present in the bat ancestor that was subsequently lost in pteropodid bats (Jebb et al., 2020). More significantly to this review, the genome-wide screenings conducted by the Bat1K project also found that bat-specific selection had occurred on several genes that are related to immunity. These changes could potentially underlie the reasoning behind the unique tolerance of viruses identified in bats. Screening identified positive selection on genes such as INAVA, which has a role in enhancing NF-κB signaling in macrophages and the B-cell chemokine CXCL13. In addition to this analysis, this study also identified 10 additional genes that have undergone positive selection in the bat ancestral lineage, including some genes involved in immune regulation and NF-κB activation (IL17D and IL1B). Collectively, from the results of these studies, it was suggested that ancestral bats have evolved immunomodulatory mechanisms that permit a higher tolerance to pathogens in comparison to most mammals, supporting the overarching theory of this review that bats possess a unique immune response against viral infection. To further support findings, the Bat1k project also completed a second genome-wide screen, but this time aimed at identifying any inactivated genes or gene losses. 10 genes from the six genomes were inactivated, two of which normally have immune-stimulating functions, again related to NF-κB. LRRC70 normally enhances NF-κB activation and response to cytokines but is inactivated in these bat species (Wang et al., 2003). Moreover, the gene IL36G, encoding a pro-inflammatory interleukin that induces the NF-κB pathway (in addition to other inflammatory cytokines) was also inactivated in bats. Both screenings for positive gene selection and inactivation identified genes involved in NF-κB signaling, suggesting this apparent altered pathway may partake in the bats immune adaptations (Jebb et al., 2020).

The Bat1K project identified that within the bat lineage, the APOBEC gene family is expanded at its locus. This is significant as this gene encodes enzymes that edit DNA and RNA and are often induced by IFN signaling, hence deeming them useful in preventing viral infection. Thereby, expansion of this gene identified in these bat lineages is likely to contribute to the unique viral tolerance mechanisms that bats display (Jebb et al., 2020). Discovery of this evidence further supports the theory that bats can tolerate viral infection much more efficiently than other mammals due to their likely unique immune systems. Viral infections often leave traces within host genomes called endogenous viral elements (EVEs), so Jebb et al. (2020) deemed it important to also screen the six bat genomes to observe if the diversity of these elements differed within the bats compared to other mammals. The project took an approach to focus separately on non-retroviral EVEs and retroviral protein-coding EVEs respectively. Three non-retroviral EVE families were identified; *Parvoviridae*, *Adenoviridae*, and *Bornaviridae* which were found within the bats as well as other mammals, in addition to a partial filovirus EVE in Vespertilionidae. Retroviral protein-coding genes were identified to be highest from beta and gamma retroviruses as predicted by the project. It was also noted that in the genomes of some of the bat species, viral envelope-encoding DNA was identified that appeared more similar to alpharetroviruses, which had previously only been identified as endogenous avian viruses. Discovery of these alpharetrovirus elements within the bat genomes indicates that they must once have been infected by these viruses that appear originally avian in origin (Jebb et al., 2020).

INTERFERON STIMULATED GENES IN BATS

Once the bat IFNs are triggered by a stimulus such as a virus, the transcription of hundreds of ISGs are stimulated. These ISGs are vital to the antiviral defence of bats, as they exert a multitude of antiviral mechanisms which collectively target almost any step in a virus life cycle (Schoggins and Rice, 2011). Despite the vast amount of information available about the IFN system of bats at the genomic level, there is still little known about the IFN-induced responses that are shaped by the transcription of ISGs. Overall, the bat interferome initially appears as standard in up-regulating core ISGs and possessing similar distributions of up or down regulated genes to other mammals along with having their own set of species-specific ISGs (Shaw et al., 2017).

Conserved Interferon-Stimulated Genes

ISG expression is known to correlate with the establishment of an antiviral state of infected and neighboring cells. There are

50–1,000 ISGs identified in humans, based on cell type and duration of IFN treatment, yet it is currently unknown how many ISGs are induced in different bat cells and within different bat species (Banerjee et al., 2020). Recent research by De La Cruz-Rivera et al. (2018) studied ISG production in the black flying fox and identified that IFN signaling in this species consists of both unique and conserved ISG expression profiles. Numerous ISGs that are known to exist in humans and other mammals have been identified in the black flying fox upon stimulation with poly I:C, including protein kinase R (PKR), 2-5-oligoadenylaye synthetase 1 (OAS1) and orthomyxovirus-resistant gene 1 (Mx1 GTPase) (Zhou et al., 2013). These three ISGs are the most studied in bats and are representative of the major antiviral pathways that are induced in an antiviral response. The OAS1 gene promoter in black flying fox cells has two IFN-stimulates response elements (ISREs), in comparison to the one ISRE observed in the human OAS1 counterpart. This could possibly play an important antiviral role in RNA infections in the bat (Zhou et al., 2013). Studies that identified IFN-stimulated transcripts from the black flying fox found that over 100 genes are induced in response to IFNα, which have previously been identified as ISGs in other mammals, suggesting strong evolutionary conservation of ISGs in bats (De La Cruz-Rivera et al., 2018). The Papenfuss et al. (2012) have also identified further ISGs in the black flying fox via transcriptome analysis including; Mx1, Mx2, OAS1, OAS2, OAS3, OAS-like (OASL), PKR, and ISG15. Induction patterns of ISGs in bat cells also appears similar to other species when induced by synthetic IFN or poly I: C. Assessing the expression profile of the black flying fox kidney cells revealed upregulation of OAS1, PKR, and Mx1 upon treatment with IFNβ and IFNλ2 (Zhou et al., 2013). *In vitro* viral infection experiments also provided evidence for induction of bat ISGs upon infection with Pteropine ortheovirus (PRV) NB which induced Mx1, OAS1 and PKR genes (Zhou et al., 2013). Upon stimulation with poly I:C, big brown bat kidney cells were found to express the transcripts for MDA5, RIG-1, radical S-adenosyl methionine domain containing 2 (RSAD2), IRF7, OAS1, IFN-inducible protein 6 (IFIT6) and Mx1. Out of all of the bat ISGs that have been examined to date, their sequence patterns appear conserved, with the exception of ISG15, which has undergone positive selection in the black flying fox (Zhang et al., 2013a). This gene has been proven to enhance the IFN response in mice, however its role in bats is yet to be studied but could suggest a similar advantageous role.

Novel Bat Interferon-Stimulated Genes

It has been hypothesized that in addition to the ISGs that bats share with other mammals, they possess a small amount of a special subset of ISGs that may have key roles in limiting viral replication and therefore contributing to their unique host-virus co-existence. Gene expression analyses have revealed a small number of novel ISGs that appear to be unique to bats, one of which is called ribonuclease L (RNASEL), which is not found in humans but is highly inducible in the black flying fox. RNASEL encodes a 2'5'-oligoadenylate synthetase-dependent RNase, which is a key protein in the antiviral response that degrades viral RNA in response to 2'5'-linked oligoadenylates produced by the OAS family of enzymes when stimulated by dsRNA from viruses (De La Cruz-Rivera et al., 2018). The induction of RNASEL in response to IFN in bats may give an extra layer of antiviral protection as knock-out experiments showed that black flying fox cells lacking RNASEL had an increased susceptibility to viral infection (De La Cruz-Rivera et al., 2018). Another study conducted on Jamaican fruit bats (*Artibeus jamaicensis*) also found an increased RNASEL level in the spleen following infection with Tacaribe virus, indicating that RNASEL induction in bats can be observed *in vivo* (Zhang et al., 2013a). In humans, only upstream proteins of OAS are induced by IFNs, whereas bats appear to induce both parts of the OAS/RNASEL pathway which is likely to induce a quicker effect to hinder viral replication before it can spread into further neighboring cells (De La Cruz-Rivera et al., 2018). Other possible unique ISGs that have been identified in the black flying fox via examination of their gene expression profiles include; EMC2, FILIP1, IL17RC, OTOGL, SLC10A2, and SLC4A1, but it is currently unknown whether these genes are expressed only in certain cells or certain species of bat (De La Cruz-Rivera et al., 2018). Additionally, molecular mechanisms of antiviral actions of these novel ISGs warrant future investigations.

Interferon-Stimulated Gene Expression in Bats

It has been recognized that in IFN-stimulated cells, bat ISGs fall into two categories that share similar early induction kinetics but possess a unique late phase decline (De La Cruz-Rivera et al., 2018). These findings are significant as this decline phase is not present in human cells, therefore bat ISG levels appear to remain elevated for longer than their human counterparts. Many studied genes had higher induction levels in comparison with human cells, suggesting that bat ISGs may provide some residual antiviral protection even when IFN signaling is returned to basal levels. This proposes possible evidence for any species-specific differences in viral susceptibility (De La Cruz-Rivera et al., 2018). It is currently unclear why only certain ISGs are temporally induced in this manner and further research is required to determine whether these are unique products of IFN induction in bats. Bat ISGs that are conserved between other species, have also been found to be expressed in higher levels than their human counterparts, which again may be another unique immune feature contributing to the viral-host relationship observed in bats (De La Cruz-Rivera et al., 2018). Overall, several novel ISGs and their atypical induction has been identified in bat cells. However, homologues of atypical ISGs expressed in human cells has not yet been studied. This could prove useful in allowing researchers to design strategies

to induce or exogenously activate antiviral pathways in other animals and humans that are infected with viruses (Banerjee et al., 2020).

FUTURE PROSPECTS

Until recently, bats have remained one of the least extensively studied mammals but have been proven as vital components in the transmission of many emerging and re-emerging diseases. With the recent COVID-19 outbreak originating from bats, it is now more important than ever to focus time and interest into bats as viral reservoirs to gain an understanding of their immunology in hope to reduce the emergence of new viruses from bats and manage their spread in future. Most bat species studied to date appear to share a myriad of immunological features with humans and other mammals. However, studies already conducted on bat immunity suggest that even though they share many key immune features to their human counterparts, bats also possess so-called "unique" immune characteristics and functional differences in the regulation of their innate immune system. It has been hypothesized that these unique immune features may allow bats to attain a symbiotic viral-host relationship whereby despite being infected with the virus, they do not display any clinical signs of disease and still retain the ability to transmit the virus to other species. Many hypotheses as to why studied bat species possess these novel immune characteristics refer to their novel behavioral features, such as flight capability, nocturnal activities, mating, and geographical distribution (O'Shea et al., 2014). These factors may all underlie or contribute to the immune mechanisms utilized by bats to give them their novel innate immune response to viruses. Although many of these immune characteristics have been unearthed, more research into their applications is required. For example, the observation that the black flying fox expresses high baseline levels of IFNα, which may provide them with an advantageous system for controlling viral replication, contradicts the reduced levels of IFNβ also detected in the Egyptian fruit bat. Conceivably, bats may manage an inimitable balance between the levels of the two key type I IFNs, aiding in their antiviral abilities. However, further research is required to make this conclusive and to comparatively assess the constitutive or inducible expression of IFNs in all bat species, not just in the black flying fox and Egyptian fruit bats.

There are many challenges to overcome when studying bats. Despite the enticing information already gained about bats and their immunity, results have only been obtained from a limited number of studies due to lack of previous information available. There is currently a limited repertoire of cell lines from very few selected bat species available for studies, which is an issue as results can cause bias toward certain bat species and hence cannot be taken as representative of the Chiroptera order as a whole. Lack of availability of material has also led to studies where viruses have been propagated and cell lines used that have been derived from unrelated or closely related bat species. Therefore, despite finding immune factors in model species experiments, namely, in the black flying fox, it is not acknowledged that this would be the case in all other species of bat. Although many *in vitro* experiments have proved successful in proving the identification of the bat IFN system and signaling, these results are not reflective of bats in the wild, as there are many discrepancies between the behaviors and characteristics of those observed in the lab in comparison to bats in their natural habitats. For example, it is critical to recognize that in the wild, bats, and other wild animals, are often infected with a multitude of viruses, bacteria, protozoans, and helminths. This heterogeneity is often lost in lab experiments. Thereby zoonotic viruses introduced to bats in natural conditions may face very different immune states to those conduced in a laboratory. Furthermore, many experiments conducted in the laboratory have used bat cells alongside human isolates of closely related viruses due to the inability to isolate most bat-borne viruses from their original source. These investigations demonstrated that infected bats do not develop the disease, causing speculations of bats as the reservoir hosts of the virus. Isolating the original bat-borne virus from the source species is required to provide a more reliable and definitive representation (Banerjee et al., 2020). Most infection studies conducted in bats or bat cells have used human isolates and virus stocks that have been propagated from non-bat cell lines. By doing so in non-natural hosts, adaptive mutations are generated in the virus which over time cause the lab cultures to no longer represent the original viral isolate from bats. To attain more representative results from bat studies, there is a desperate desire to obtain direct viral isolates from bat hosts in the wild, which poses an obvious challenge for researchers and data collection.

One of the main limiting factors in the study of bats is their incomplete genomic annotation. The available genomes are limited to few bat species which presents a narrow comparison window, although the recent annotations of six bat genomes by the Bat1K project now shows promising potential, with plans to also annotate genomes of further bat species (Jebb et al., 2020). Application of genome-scale transcriptomics, genomics, and metabolomics in diverse bat species will highlight the true uniqueness against multiple zoonotic viruses. Despite bats proving to be important hosts of zoonotic viruses, there is still very little known about their host-virus relationships, largely because there are very few bat colonies available for experimentation and limited availability of reagents. With developed methods and expertise being developed, bat antiviral responses may be explored in much better detail, allowing us to gain an understanding of how bats interact with the virus and how they are transmitted between species (Schountz, 2014). Based on the previous work conducted on innate immunity in bats, notably their IFN response, bat immunity appears a highly promising study-model in providing useful insights into this fertile research area. Due to previous lack of material availability and the potential

applications of the bat innate immune response, an exciting opportunity for research is now obtainable to explore the bat IFN response in more detail and gain a full understanding. With the field of bat immunology and virology now progressing at a faster rate than before, prospects for research into bat innate immunity and their unique host-virus relationship will aim to provide useful in determining the extent of their immune capabilities and their role as a key zoonotic host. Furthermore, via discovering the novel adaptations of bat immune systems, the current understanding of the human immune response may be redefined and possibly utilized when applied to other species in preventing pathology of disease.

AUTHOR CONTRIBUTIONS

MM and EC perceived the idea and wrote and revised the manuscript. All authors contributed to the article and approved the submitted version.

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