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Review article

Antiviral responses against chicken respiratory infections: Focus on avian influenza virus and infectious bronchitis virus

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ABSTRACT

Some of the respiratory viral infections in chickens pose a significant threat to the poultry industry and public health. In response to viral infections, host innate responses provide the first line of defense against viruses, which often act even before the establishment of the infection. Host cells sense the presence of viral components through germinal encoded pattern recognition receptors (PRRs). The engagement of PRRs with pathogen-associated molecular patterns leads to the induction of pro-inflammatory and interferon productions. Induced antiviral responses play a critical role in the outcome of the infections. In order to improve current strategies for control of viral infections or to advance new strategies aimed against viral infections, a deep understanding of host-virus interaction and induction of antiviral responses is required. In this review, we summarized recent progress in understanding innate antiviral responses in chickens with a focus on the avian influenza virus and infectious bronchitis virus.

1. Introduction

Chicken meat and egg are the most consumed sources of animal protein at the global level. The global production of poultry meat and eggs is around 100 and 73 million tons, respectively. The poultry industry has a significant impact on the economy. For example, this industry, including farming and processing, contributes around $7 billion to Canada's gross domestic product, which demonstrates its importance. However, the poultry industry is constantly at the risk of infectious diseases, including viral infections. Viral infections are major concerns in the poultry industry worldwide, causing drastic economic losses. Some high-priority, economically important poultry viruses, such as the avian influenza virus cause enormous socio-economic impacts on public health. The expanding of both human and livestock populations, changes in the farming system with the goal of increasing production and financial benefit, the globalization of livestock farming increase the chance of the occurrence of emerging infectious disease and their spread. Therefore, there is a vital need to gain a better understanding of sources and causes regarding the emergence and spread of viral infections in poultry which will improve our strategies to control their spread and possible intra-species transmission.

There are several high-priority, economically important poultry viruses, including the avian influenza virus (AIV) and infectious bronchitis virus (IBV).

Avian influenza virus infection is a worldwide spread and highly contagious disease which can affect a wide variety of birds and mammals. Avian influenza virus (AIV) is an enveloped, negative-sense single-stranded segmented RNA virus in the family of *Orthomyxoviridae* type A influenzas [1]. Based on clinical signs in chickens, AIVs have been classified into two pathotypes: highly pathogenic avian influenza (HPAI) viruses and low pathogenic avian influenza (LPAI) viruses [2]. Among 18HA subtypes of AIVs, only the H5 and H7 subtypes evolve into a highly pathogenic form of the disease. HPAI viruses cause severe outbreaks and mortality in chickens, whereas LPAI viruses usually cause mild respiratory disease [3]. The outbreaks cause a decline in the production and slaughter of birds. Enhanced biosecurity measures, surveillance, stamping out, and quarantine of infected and contact chickens are the cornerstone measures for control of AIV. AIV infected poultry can also represent a source of human infections depending on the subtype of AIV [4].

Avian infectious bronchitis virus (IBV) is a group 3 coronavirus established in countries with an intensive poultry industry. It is a highly

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contagious pathogen with geographically distinct strains [5]. In chickens, the virus is transmitted mainly through aerosols and ingestion of feces or contaminated water and feed. The resulting disease, infectious bronchitis (IB), is predominantly mild-severe respiratory disease. Furthermore, the virus can infect the other part of the respiratory system, including the lung and air sacs. Then, depending on the virus strain, the virus can be found in other epithelial cells, such as oviduct and kidney epithelial cells, and causes the infection in other tissues. Some of these strains, including variant D388, known as QX genotype, or the Massachusetts (Mass) serotype cause pathological lesions in chicken oviducts, which leads to cystic oviduct formation in young pullets and subsequently false layer syndrome in the peak of production [6–8].

Even though extensive research has been conducted in the last several decades to improve surveillance methods, biosecurity, and vaccines for the control of viral infections in poultry, recent outbreaks of both viral infections have provoked notable concerns about measures available for control of these infections. In conjunction with the research efforts focused on developing effective vaccines and prophylactic strategies, there is a need to explore novel approaches to tailor favorable host innate responses, as a part of a strategy to induce rapid innate antiviral responses against viral infections. The induction of effective innate antiviral responses in chickens is a new strategy that recently gained increasing attention. Innate responses provide the first line of defence against invading viruses. To induce proper innate antiviral responses, it becomes of necessity to have a better understanding of the intracellular pathways which are triggered by the viruses. Chicken airway epithelial cells are the primary target of respiratory viral infections that play a significant role in the induction and coordination of the innate responses. These cells build a barrier as the first line of defense by isolating the lumen, and luminal surfaces from basolateral surfaces. Chicken tracheal epithelial cells are able to induce antiviral responses against viral infections and affect the functions of neighboring cells, such as macrophages. The cross-talk between different host cells directs the immune responses and the outcome of the infection. Therefore, inducing suitable responses could be a potent and efficacious strategy to induce host innate responses [9]. There are some pieces of evidence that AIV can replicate in chicken tracheal epithelial cells with increasing virus titer in a short period of time, demonstrating the susceptibility of tracheal epithelial cells to AIV infection [9–11]. Innate antiviral responses against viruses include the sensing of viral proteins and nucleic acids, production of cytokines, and chemokines [12]. Some components of innate antiviral responses against AIV and IBV are described in this review (see Fig. 1).

2. Innate immune responses

The innate system is the first line of defense against viral infections. This system contains different components to provide a barrier against microbes. Rapid and non-specific innate responses can reduce or inhibit viral replication at the site of infection. The mucus produced by airway epithelial cells maintains a physical barrier against microbes. The mucus contains cells and cell debris as well as mucin. Mucin is a heterogeneous glycoprotein produced by different types of airway epithelial cells, such as basal cells, goblet cells, and ciliated cells. Two types of mucin are present in the respiratory system; a secreted form (for example MUC5AC and MUC5B) and a form present on the cell surface acting as a receptor (for example, MUC1, MUC4, MUC11, MUC13) [13,14]. Mucin can capture viruses because of its viscosity and is known as a physical barrier.

In the lower part of the respiratory system, lectin, which can bind to carbohydrates, is present instead of mucin. Three classes of lectins have been identified in mammalian species; C-type lectin, S-type lectin, and pentraxins. The C-type lectin subclass has a carbohydrate recognition domain, and also binds to influenza virus, inhibiting its attachment. Collectin, another subclass of C-type lectin, inhibits influenza virus infection by interaction with the HA antigen of the virus and inhibiting HA activity [15].

However, when the virus passes these preliminary physical barriers, other cells in the respiratory system, such as macrophages and lymphocytes interact with the virus. These are the primary cells that encounter AIV. Following either infection of the respiratory system or vaccination, antigens will be taken up by some phagocytic cells, such as macrophages or dendritic cells (DC). Phagocytic cells are potent antigen-presenting cells that activate T cells. In addition, phagocytic cells are located beneath epithelial surfaces. In mice, both conventional and plasmacytoid dendritic cells (cDC and pDCs, respectively) are located in the respiratory tract, parenchyma of the respiratory system, and trachea.

Moreover, alveolar macrophages are located in alveolar spaces. In mammalian species, alveolar macrophages play a key role in protecting tissues with large surface areas against pathogens. Alveolar macrophages in the alveoli space, phagocytize cell debris of influenza virus-infected cells to limit the spread of the virus. In addition, the activated macrophages secrete nitric oxide synthase 2 (NOS2) [16]. Notably, this activity should be tightly regulated, since large amounts of nitric oxide synthase 2 contribute to the pathogenicity of avian influenza virus [17,18].

Antigen-presenting cells (APC) take up antigens and subsequently migrate into bronchus-associated lymphoid tissue (BALT) areas [19,20]. In humans, cDCs are present beneath epithelial cells and above the basal membrane. DCs play a vital role in the initiation phase of immune responses to influenza virus infection and can recognize virions and debris of infected cells. Following the sensing or capturing of viral antigens by cDCs, these DCs migrate to lymph nodes in a CCR7-dependent manner to present the viral epitopes to CD4 + T cells via major histocompatibility complex (MHC) class II [16,21]. Moreover, DCs also present viral epitopes to CD8 + T cells via cross-presentation [22]. The activity of cytotoxic CD8 + T cells is crucial to eliminate influenza virus infection. Transportation of viral antigens from the site of infection to draining lymph nodes by DCs influences the activation of T cell subsets. CD103 + DCs are resistant to influenza virus infection. Therefore their existence is critical for the transportation of viral antigens. It has been shown that the expression of interferon-induced transmembrane protein (IFITM)3 in CD103 + DCs ensures their resistance to influenza virus infection [23]. However, some tissue-resident DCs are susceptible to influenza virus infection. As a result, they cannot migrate to lymph nodes to present viral epitopes to T cells. In the virus-infected DCs, inflammasome and caspase-1 are activated following the infection, leading to the production of interleukin (IL) −1 beta, and induction of pyroptosis. Pyroptosis is a form of host cell death triggered by various stimuli, including infection with pathogens and activation of the inflammasome and caspase 1 pathway [24]. Following IL-1 production, other DCs which are known as bystander DCs, move toward the site of infection to capture virus particles and migrate to lymph nodes [25].

In chickens, the trachea is able to mount immune responses against pathogens. Following initiation of immune responses by the chicken trachea, the infiltration of cells of the immune system, including lymphocytes and macrophages, has been previously observed [26–29]. In chickens, APCs, such as subepithelial phagocytes and interstitial macrophages, are not present on the external surface of the chicken respiratory system and do not migrate toward the lumen of the respiratory tract. In fact, viral antigens are transported to APCs by the epithelial layer, which highlights, the essential contribution of airway epithelial cells in host responses against AIV in chickens [19,30].

3. Virus life cycle, cell attachment, and virus entry

3.1. Avian influenza virus:

The attachment of viral glycoproteins to sialic acid (SA) glycoproteins on the surface of host cells is the first step of infection. Sialic acid

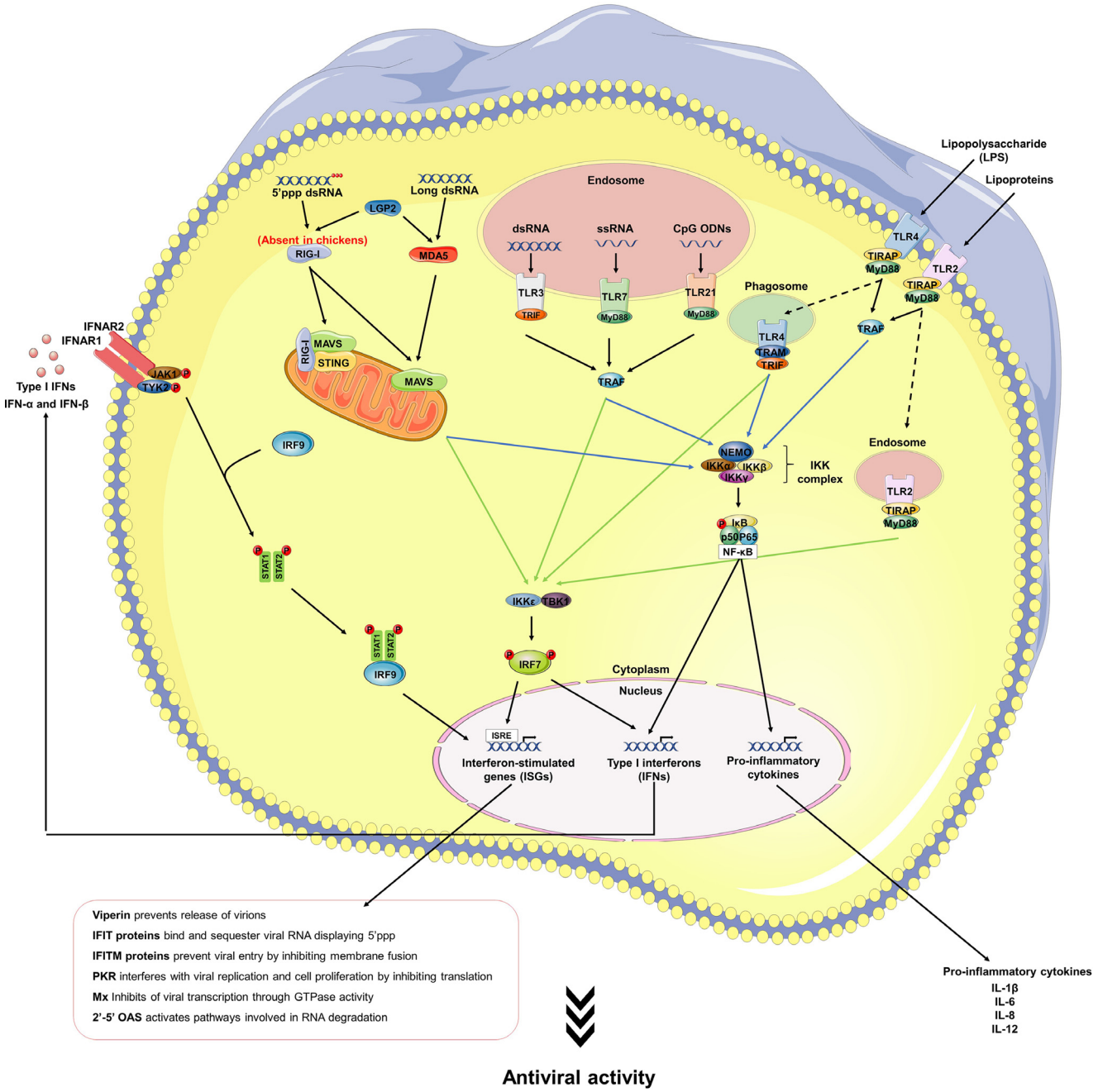

Fig. 1. Summary of antiviral signaling pathways in chickens. Chicken antiviral responses rely on the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs). Viral double-stranded RNA (dsRNA) is detected by chicken melanoma differentiation-associated gene 5 (MDA5) or laboratory of genetics and physiology 2 (LGP2), which triggers downstream signaling mediated through mitochondrial antiviral-signaling protein (MAVS). Endosomal Toll-like receptors (TLRs), TLR3, TLR7 and TLR21 can recognize dsRNA, ssRNA and cytosine-guanosine oligodeoxynucleotides (CpG ODNs), respectively. TLR3 signals through adaptor protein TIR-domain-containing adapter-inducing interferon (TRIF) and TLR7 and 21 signal through myeloid differentiation primary response 88 (MyD88). Surface TLR2 and 4 also signal through MyD88. However, when present in the phagosome, TLR4 signals through adaptor protein TRIF and TRAM (TRIF-related adaptor molecule). These adaptor molecules then activate the transcription factors interferon regulatory factor (IRF)7 and nuclear factor kappa B (NF-κB), by coordinating the assembly of multi-protein complexes. Upon activation, IRF7 and NF-κB induce transcription of type I interferons (IFNs), pro-inflammatory cytokines, and interferon-stimulated genes (ISGs). Additionally, secreted type I IFNs can activate Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway through type I IFN receptor binding. Activated JAK/STAT leads to the phosphorylation of STAT1 and STAT2 molecules, results in recognition of the IFN-stimulated response element (ISRE) and transcription of ISGs. Activation of these various pathways leads to the induction of an antiviral state. This figure was created using Servier Medical Art templates, which are licensed under a Creative Commons Attribution 3.0 Unported License; https://smart.servier.com.

moieties on cell surfaces are the primary receptors for viral entry [31]. However, it has been shown that macrophage mannose receptor (MMR), macrophage galactose lectin (MGL), and C-type lectin receptor are necessary for virus uptake in macrophages. Indeed, these receptors act as co-receptors with sialic acid receptors [32]. Carbon sugars of sialic acid moieties are connected to basal sugar chains of glycoproteins in the cell membrane through α2,3 or α2,6 (SAα2,3 or SAα2,6) linkages.

Avian and human origin influenza viruses have a varying affinity to different SA moieties. In fact, the hemagglutinin glycoprotein binds to

the host cell surface depending on the presence of specific sialic acid moieties on host cells. As a result, the viral hemagglutinin glycoprotein affects the host range of different influenza viruses and is known as a determinant of host susceptibility. Changes in the amino acid sequence of hemagglutinin affect the sialic acid receptor specificity and pathogenicity of the virus [33]. The HA of human origin viruses commonly binds to SA moieties with α2,6 linkages, while the HA of avian origin viruses commonly binds to SA moieties with α2,3 linkages. In addition, the patterns of α2,3 and α2,6 sialic acid expression differ in a variety of mammalian and avian species [34–36]. In humans, tracheal epithelial cells primarily contain SA moieties with α2,6 linkages, whereas the tracheal epithelium in chickens and pigs expresses both SA moieties with α2,6 and α2,3 linkages [35–37]. However, the expression pattern of SA moieties is varying among different cells of the epithelium in chickens. For example, chicken tracheal ciliated cells mostly express α2,3-linked SA, while α2,6-linked SA is expressed in goblet cells. Basal cells express both α2,3-linked SA and α2,6-linked SA [11].

3.2. Infectious bronchitis virus:

Chickens rapidly transmit IBV through several channels, which is primarily a result of the proximity and shared resources of the birds. In addition, evidence of vertical transmission has also been reported [38]. In a typical respiratory infection, when inhaled, the positive-sense single-stranded RNA virus will bind and enter host tracheal epithelial cells, which requires interactions between cell surface receptors and viral structural proteins involved in attachment [39]. The spike protein of coronaviruses is responsible for attachment and entry into host cells. The S1 subunit is mainly responsible for attachment to receptors on host cells, while the S2 subunit is responsible for fusion to virus and host membranes.

The tropism of viral infections to different cells of epithelium could be related to the distribution of viral receptors. For example, IBV is able to infect ciliated cells and goblet cells, while basal cells are resistant to the infection with IBV. The basal cells, multipotent stem cells, play a critical role following the infection and cell death in the ciliated and goblet cells [40].

Once the virus enters the host cells, it replicates in tracheal cells, which leads to multiple changes in the epithelial mucosa: ciliary loss, degeneration, necrosis of epithelial cells and the infiltration of inflammatory cell. All of the aforementioned factors largely contribute to the pathology of IBV. This dramatically impairs the mucociliary apparatus, which, in itself, is a mechanical defense system that acts to physically clear IBV. Eventually, IBV can undergo secondary replication and infect tissues such as upper respiratory tract nose, lungs, air sacs, as well as kidneys [41].

4. Pattern recognition receptors (PRRs)

The innate system provides the first line of defense against potentially lethal pathogens. In the context of viral infections, viral component sensing, and cytokine production result in downstream signaling to activate additional components of the immune system. Chickens are able to detect the presence of viral components through germinal-encoded pattern recognition receptors (PRRs), which identify pathogen-associated molecular patterns (PAMPs). PAMPs are highly conserved molecular structures that are present in pathogens and microbes, including lipopolysaccharides (LPS), microbial lipoprotein, and peptidoglycans presented only in microorganisms [13]. PRRs are expressed on host cells including the cells of the immune system and other host cells. Chicken PRRs can recognize PAMPs presented in AIV and IBV [42]. Three main families of PRRs include Toll-like receptors (TLRs), Retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). These families act alone or together to induce intracellular signaling, pro-inflammatory cytokines, anti-apoptotic factors, type I interferons (IFNs), and host defense peptide (HDP) production [43].

4.1. Toll-like receptors

Overall, TLRs are the most studied member of PRRs in chickens. Currently, 10 human TLRs (huTLR), 13 murine TLRs, and 10 chicken (*Gallus gallus*) TLRs (chTLR) have been characterized. Chicken TLRs include chTLR1A and chTLR1B, chTLR2A and chTLR2B, chTLR3, chTLR4, chTLR5, chTLR7, chTLR15, and chTLR21 [42]. The adaptor molecules and signaling pathways are initiated as a consequence [44,45]. TLR-PAMP interaction results in the activation of intracellular signaling pathways, and subsequently, the production of pro-inflammatory cytokines, such as interleukin (IL) −6, IL-1β and type I interferons (IFNs) [46,47]. Recent studies have shown the critical role of TLRs in initiating an early response against AIV in chickens [9,48–50].

4.1.1. TLR2

TLR2 is expressed in different cells and tissues in chickens, including macrophages, tracheal cells, thrombocytes, erythrocytes, spleen, and cecal tonsils [9,47,50–52]. Previous studies demonstrated that the activation of cells with different PAPMs leads to the up-regulation of TLR2. Stimulation of TLR2 with its cognate ligands results in the induction of intracellular signaling pathways and the initiation of the innate responses, which interfere with the replication of pathogens within host cells. Several studies in chickens using *in vitro, in ovo,* or *in vivo* models demonstrated the induction of antiviral responses following TLR2 stimulation in chickens. The activations of TLR2 with Pam3CSK4, TLR2 ligand, results in the expression of pro-inflammatory cytokines. Increased expression of IL-1β in macrophages induced with Pam3CSk4 has been coinciding with the reduction in AIV replication [50]. In addition, enhanced expression of IL-1β was observed in chicken tracheal cells following TLR2 ligand treatment. IL-1β can induce NO synthesis, the proliferation of T and B cells and recruitment of lymphocytes, macrophages, and heterophils and finally can inhibit the spread of infection in the host [53]. Activations of TLR2 with Pam3CSK4 enhances the expression of interferon regulatory transcription factor (IRF)1, IRF7, type I interferons (IFNs), IFN-ɣ, and the interferon-stimulated genes (ISGs). In chickens, IRFs involve in antiviral responses by the regulation of interferon responses. Pam3CSK4 is able to induce a wide array of ISGs in chickens, including 2′-5′-oligoadenylate synthetase (OAS) and interferon-induced transmembrane protein (IFITM)5, protein kinase R (PKR) and viperin. The induction of type I IFN and ISG expression following TLR ligand stimulation can limit AIV shedding from infected chickens since IFNs and ISGs play an essential role in interfering with AIV infection. Barjesteh et al. demonstrated that the pre-stimulation of macrophages with Pam3CSk4 reduces H4N6 AIV replication [50]. In chickens, intranasal, and intramuscular administration of TLR2 ligand, significantly reduces oral and cloacal AIV shedding. The intranasal treatment of Pam3CSK4 is more effective than the intramuscular administration. In chickens, intranasal treatment with Pam3CSk4 results in the up-regulation of IFN-β and ISGs (PKR, viperin, and melanoma differentiation-associated protein 5 (MDA5) in trachea and down-regulation of IFN-β and viperin and up-regulation of PKR and MDA5 in cecal tonsils. These data suggested that intranasal treatment may locally stimulate respiratory epithelial cells to induce antiviral responses and subsequently limit AIV replication [47].

4.1.2. TLR3

TLR3 is activated by double-stranded RNAs (dsRNA), which forms during the replication of AIV and IBV. The activation of TLR3 leads to the up-regulation of cytokines, such as IL-6, IL-12 in chickens [46]. IRF7 is activated following the activation of TLR3 which results in an increased production of type I IFNs in chickens. TLR3 expression is increased following viral infections, such as H5N1 avian influenza infection [54] as well as following the activation of TLR3 by its cognate or

non-cognate ligands. A previous study demonstrated the enhanced TLR3 expression in different chicken cells and subsequent induction of antiviral responses following TLR2, 4, and 21 ligand treatments [47,49].

Poly(I:C), TLR3 ligand, is a dsRNA molecule that has been widely employed in chickens as an antiviral agent in prophylactic strategies. The prophylactic treatment of chickens with polyI:C, prior to influenza virus infection, significantly reduces influenza virus replication and virus shedding and has the ability to increase host immunity against AIV. It is suggested that IFN-α and IL-8 expressions are correlated with immunity conferred by poly I:C [55]. The anti-influenza property of poly I:C may be attributed to the unique signaling pathway utilized by TLR3, which signals solely through the TIR domain-containing adaptor inducing IFN-β (TRIF) pathway and promotes the production of type I IFNs, IFN-α and IFN-β [55,56]. Barjesteh et al. indicated that treatment of chicken tracheal epithelial cells (cTECs) with polyI:C reduces the replication of AIV in cTECs [9]. In contrast, BX795 and celastrol, inhibitors of IRF7 and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) pathways, increase AIV replication in polyI:C treated cells demonstrating the role of IRF7 and NF-kB signaling pathways in the induction of antiviral responses post TLR3 stimulation. PolyI:C induces IL-1β, IL-6 and IL-8 expression in cTECs [57]. The induction of pro-inflammatory responses in cTECs through the NF-κB pathway following polyI:C treatment can recruit macrophages to the site of infection. The chemotactic effects of supernatants from cTECs treated with polyI:C on chicken macrophages, indicated increased macrophage migration toward TEC supernatants. In addition, it has been demonstrated that supernatants from cTECs treated with TLR ligands can contain IFNs, ISGs or other active antiviral components that interfere with AIV replication in other host cells, such as chicken macrophages [9]. Moreover, Ahmed-Hassan et al. showed that *in ovo* treatment of eggs with dsRNA activates the innate responses against H4N6 LPAIV infection through increasing TLR3 expression and type I IFN production as well as macrophage recruitment. In addition, dsRNA elicits antiviral responses against LPAIV correlating with type I IFN activity in macrophages *in vitro* [4].

4.1.3. TLR4

TLR4 is expressed in most chicken cells, including macrophages, heterophils, B cells, as well as in tissues including the trachea, the spleen, and bursa of Fabricius [58,59]. Bacterial lipopolysaccharide (LPS) is the main component of the outer wall of all gram-negative bacteria which is recognized by TLR4. TLR4 on the surface of cells interacts with three different extracellular proteins: LPS binding protein (LBP), CD14 and, myeloid differentiation protein 2 (MD-2) [60]. TLR4 ligand activates two downstream intracellular signaling routes: myeloid differentiation primary response gene (MyD88) dependent and independent pathways in chicken macrophages [61]. Prophylactic treatment of chickens with LPS, TLR4 ligand, prior to influenza virus infection, significantly reduces virus shedding and has the ability to increase host immunity against AIV [55,62]. Barjesteh et al. demonstrated that treatment of chickens with LPS prior to AIV infection reduces AIV shedding. LPS from *Escherichia coli* 026:B6 reduces cloacal virus shedding and both low and high doses of LPS could significantly reduce oral shedding of H4N6 AIV. Moreover, TLR4 ligand induces the expression of genes involved in antiviral responses such as type I IFN and ISGs in the chicken trachea and cecal tonsils. The results of this study raise the possibility of treatment of chickens with LPS as antiviral agents [47]. Moreover, pre-treatment of macrophage by LPS showed a significant reduction in H4N6 AIV replication. The study revealed that LPS can activate macrophages and increase in expression of IL-1β and IFN-ɣ in stimulated macrophages concurrent with the reduction in viral replication. It is assumed that this reduction is mediated by reinforcement of macrophages antiviral responses, and TLR4 ligand may interfere with AIV replication through IFNs induction [50]. It is discovered that the treatment of embryonated chicken eggs with LPS reduces AIV infection. Following embryonic treatment, the expression of IL-1β increases in the chorioallantoic membrane (CAM), which may recruit other cells, such as macrophages, to the site of infection. In addition, the expression of IFN-ɣ is upregulated after TLR4 ligand treatment in CAM, which may play a role in the reduction of the AIV titer [63]. However, the direct antiviral activity of IFN-ɣ against AIV in chickens has not been characterized. In addition, cTECs treated with LPS show a reduction in replication of AIV. The blockage of IRF7 pathway with BX795 in LPS treated cTECs, results in increased AIV replication, indicating that IRF7 pathway plays a significant role in induction of antiviral response through TLR4 ligand stimulation [57]. Furthermore, treated cells with TLR4 influence the functions of neighboring cells. For example, Barjesteh et al. demonstrated the effects of supernatants of tracheal cells treated with LPS on macrophages. These cells can activate macrophages and increase the production of NO by chicken macrophages [49]. In addition, treated cells with TLR4 liagand are able to communicate with chicken macrophages in which co-culture of treated tracheal cells with macrophages results in the limitation of AIV in macrophages [9,57].

4.1.4. TLR7

TLR7 binds to viral single-stranded ribonucleic acid (ssRNA) or synthetic analogs (i.e. resiquimod, imiquimod, gardiquimod, and ioxoribine) which and activates the myeloid differentiation primary response gene 88 (MyD88)-mediated pathway. Activation of this pathway leads to the production of pro-inflammatory cytokines interleukin (IL) 1β and IL-6 [64,65]. Stewart et al. demonstrated the antiviral properties of the TLR7 ligand, loxoribine, in chickens demonstrating its antiviral properties of loxoribine. The treatment of primary chicken splenocytes with loxoribine resulted in the induction of interferons-α, -β, and -ɣ, and ISGs including PKR and Mx [48]. Additionally, resiquimod, a TLR7 ligand can increase IL-1β production in chicken macrophages, which leads to stimulation of antiviral responses against AIV. The induction of antiviral response against AIV by TLR7 ligands is attributable to IL-1β production and not to the NO production [66]. In addition, after treatment of embryonated chicken eggs with R848, a TLR7 ligand, IL-1β is upregulated in CAM, which may recruit macrophages to the CAM. This recruitment may be the source of iNOS in the CAM and the cause of the inhibition in AIV replication [63]. R848 has been used *in vivo* in chickens resulting in a significant reduction of cloacal and orad AIV shedding [47].

4.1.5. TLR21

TLR21 recognizes microbial DNA containing unmethylated cytosine-guanosine deoxynucleotide (CpG) motifs in avian species [67]. CpG oligodeoxynucleotides (ODNs) acts as a potent immunomodulatory agent in chickens by inducing innate responses against pathogens. CpG ODNs can be used for the stimulation of the TLR21 signaling pathway in chickens resulting in the up-regulation of mRNA expression of pro-inflammatory cytokines, and the recruitment cells of the innate system, such as macrophages [68]. Recently, the *in ovo* delivery of CpG ODNs was shown to protect chickens against many bacterial and viral infections by activating the TLR21 signaling pathway. This pathway resulted in increased recruitment of macrophages, a cluster of differentiation (CD)8α + and CD4 + T lymphocytes, and an up-regulation of interferon (IFN)-γ mRNA in the respiratory tract of the chickens [69]. In a previous study, chicken embryos were inoculated with CpG ODN and challenged with virulent IBV. The results showed that there was a significant differential up-regulation of IFN-γ, IL-8 (CXCLi2) and macrophage inflammatory protein (MIP)-1β genes and suppression of IL-6 gene expression being associated with inhibition of IBV replication in pre-treated lungs tissue retrieved from embryos [70]. In addition, the transcriptional analysis revealed that CpG ODN is able to induce the expression of IL- 1β, IFN-γ, IRF7, and IFN-β in macrophages, which might play a role in control in viral replication in these cells [50]. The delivery of CpG ODNs *in ovo* in chickens before hatching day

significantly reduces H4N6 AIV replication in the chicken lungs after they hatch [69]. These reductions are associated with enhanced NO production and macrophage recruitment [69]. Furthermore, the treatment of embryonated eggs treated with CpG ODNs leads to the reduction of AIV replication in embryonated eggs which can be correlated with the induction of type I IFNs and ISGs, such as OAS and IFITM5 in CAM [63].

Besides, CpG ODNs are able to induce antiviral responses against AIV replication *in vivo* and *in vitro* [50,55]. Barjesteh et al. showed the ability of CpG ODNs to induce antiviral responses in chicken macrophages with an increase in the expression of IRF1 and IRF7 genes, type I and II interferons and ISGs, such as 2′-5′ OAS. In macrophages stimulated with CpG DNA, reduction of AIV titer was detected, which was correlated with the up-regulation of IL-1β and IFN-ɣ. CpG ODN can be used prophylactically in chickens to enhance host immunity against AIV [50]. Intramuscular injections of either low or high doses of the TLR ligands CpG ODN leads to a reduction in virus shedding. It is suggested that this reduction is mediated by the induction of type I IFNs, ISGs and IFN-ɣ. However, different classes of CpG ODNs have varying effects on chickens [50]. Chicken macrophages respond differently to different types of CpG ODNs in a sequence-specific manner [70]. The administration of different classes of CpG ODNs results in different levels of induced antiviral responses in chickens. The class B CpG ODNs, including 2007 and 1826 are more effective in reducing viral shedding from infected chickens than class A (2216) or class C (2395) CpG ODNs [47]. Consequently, different degrees of antiviral effects within class B CpG (2007 and 1826) were observed in which CpG 1826 is more efficacious to induce antiviral responses compared to CpG 2007 [47].

4.1.6. TLR ligands as vaccine adjuvants in the context of AIV infection

In addition to the possible application of TLR ligands as immunostimulatory molecules in prophylactic approaches for control of AIV, TLR ligands can be used as vaccine adjuvants or antimicrobial agents in chickens [55,71]. TLR 2, 3, 4, 5 and 21 ligands have been shown to be efficacious adjuvants for AIV vaccines in chickens [71,72]. PolyI:C and CpG combination adjuvant enhances antibody-mediated and cell-mediated immune responses against AIV antigens. A possible mechanism may be attributed in part to the synergistic IFN-ɣ response in chicken monocytes stimulated with poly I:C and CpG combinations. PolyI:C and CpG adjuvants may result in the synergistic induction of IFN-ɣ upon acting directly on tissue-resident macrophages and dendritic cells soon after immunization. Moreover, polyI:C and CpG ODN combination adjuvant can promote the induction of a robust T-cell response [72]. The induction of T-cell responses is highly desired in an AIV vaccine, as effector T-cells have been shown to protect the host against lethal H5N1 AIV infections in chickens [73]. Therefore, polyI:C and CpG ODN combination adjuvant improves the efficacy of the inactivated whole virus AIV vaccines by enhancing T-cell mediated responses [72].

Furthermore, vaccination of chickens with virosomes adjuvanted with CpG ODN can induce high hemagglutination inhibition (HI), systemic and mucosal antibodies, and can significantly decrease virus shedding after virus challenge [74]. The comparison of the immunogenicity of avian influenza virosomes with or without the inclusion of recombinant chicken interferon-gamma (rChIFN-ɣ) or CpG-ODN in chickens revealed influenza virosomes adjuvanted with CpG-ODN induces higher HI antibody titers, as well as IgG and IgA serum antibody responses compared to non-adjuvanted virosome. Moreover, the virosomes and CpG ODN formulation stimulate an antigen-specific spleen cell proliferation and IFN-ɣ expression. It is suggested that that virus-specific antibody- and cell-mediated responses can be induced in chickens immunized with virosomes, and these responses can be enhanced by incorporating CpG-ODN in the virosome vaccine formulation [75].

The encapsulated TLR ligands in biodegradable poly (D,L-lactide-co-glycolide) polymer nanoparticles (PLGA NPs) have the ability to conserve prolonged innate responses through up-regulation of IFN-γ and IL-1β [76]. Consequently, stimulation of the innate immune system with a slow release of TLR ligands from the polymers can enhance antigen-specific immune responses and may be applied as a vaccine or antimicrobial agent in the future [76].

4.2. RIG-I like receptor

The RIG-I like receptors (RLRs) are cytosolic PRRs that mediate the innate antiviral responses. The RLR family consists of three members: retinoic acid-inducible gene-I (RIG-I), melanoma differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2) [77]. RIG-I and MDA5 activate IFN regulatory factor 7 (IRF-7) and NF-kB pathways through the adaptor protein mitochondrial antiviral-signaling protein (MAVS; also known as VISA, IPS-1, and CARDIF) [77]. Activation of MAVS, which is an N-terminal caspase recruitment (CARD) domain, results in the secretion of proinflammatory cytokines and type I IFNs [78].

In mammals, RIG-I is the main cytosolic PRR for detecting influenza A virus infection [79]. The RIG-I gene has not been identified in chickens. AIV infections in chicken cells are detected by MDA5, and type I IFN induction involves chicken LGP2, MAVS, and IRF7 [78]. Despite the lack of RIG-I in chickens, MDA5 compensates for the missing gene [42]. However, a previous study suggested that the lack of RIG-I in chickens leads to inefficient antiviral innate immune response induced by this pathway [80]. In contrast, Liniger et al. showed that MDA5 functionally responsible for sensing AIV in the absence of RIG-I. In addition, it has been revealed that chicken LGP2 is a positive regulator of MDA5 signaling, similarly to those in the mammalian RLR pathway [78]. There seems to be contrary evidence that the lack of RIG-1 in chickens does not affect the susceptibility of chicken to AIV infection. Chicken MDA5 (chMDA5) expression is upregulated in response to influenza infection and following IFN activation of cells, but knockdown of chMDA5 expression does not appear to impact influenza virus replication.

Previous studies evaluated IBV infection and gene expression in chickens cells, and it was shown that IBV infection induces mRNA expression of MDA5 and LGP2 [81,82]. Chicken cells transfected with domestic goose RIG-I exhibited increased IFN-β activity after IBV infection. This indicates that chicken MDA5 is functionally active during IBV infection, but the absence of RIG-I may increase the susceptibility of chickens to IBV infection. IBV has been shown to cleave MAVS. Activated MAVS can recruit downstream IRF7 and NF-κB, leading to the rapid production of type I IFNs and pro-inflammatory cytokines. The cleavage of MAVS, which occurs at the early stage of the infection, allows IBV to escape MDA5-mediated antiviral responses and to disrupt the activation of the host antiviral response [83].

There are several mediators that regulate RLR signaling pathway, including members of the tripartite motif (TRIM) family (such as TRIM25 and TRIM27), Riplet, Zinc-finger antiviral protein (ZAP) and stimulator of interferon gene (STING). Riplet and TRIM27 are missing in the chicken genome.

TRIM25 mediates the ubiquitination of the CARD domain of RIG-I which subsequently leads to RIG-I binging to the adaptor protein MAVS, activation of the RLR signaling and type I IFN production [84]. RIG-I is not present in chickens, but previous studies demonstrated that the role of TRIM25 is influenza virus infection and type I IFN production. Chicken TRIM25 (chTRIM25) is able to bind to the NS1 protein of avian influenza viruses. It has been shown that the binding of NS1 protein to chicken TRIM25 reduces type I IFN production. In addition, TRIM25 plays a critical role in the establishment of antiviral responses in chickens. In fact, the binding of the NS1 protein of the influenza virus to TRIM25 interferes with the induction of type I IFNs [85].

Chicken ZAP, like mammalian species, has an antiviral activity that directly interacts with RNA viruses and interferes with the virus translation. In mammalian species, there are two short and long

isoforms of ZAP. However, the only long isoform of ZAP has been identified in chickens [86]. In mammalian species, the shorter isoform of ZAP is known as the regulator of RIG-I signaling. Therefore, the function of chicken ZAP as a positive regulator of RLR signaling pathway is not clear. Chicken Zap is identified as an ISG. The expression of chicken ZAP gene is induced by viral infections, such as avian influenza virus and infectious bursal disease virus, and viral mimics in chickens [86].

In mammalian species, STING is known as a cytosolic DNA recognition receptor which induces type I IFN signaling and antiviral responses following the DNA or RNA recognition. SING and RIG-I pathways are interconnected. STING as an adaptor molecule STING interacts with MAVS to enhance antiviral responses. In fact, STING is the main player to coordinate cross-talks between RNA and DNA sensing pathways [87]. In the absent of RIG-I in chickens, STING plays a critical role in the induction of type I IFNS in chickens. Chicken STING is able to interact with chicken MDA5 and it may have a role in MDA5 signaling in chickens [88].

4.3. NOD-like receptors

NOD-like receptors (NLRs) are cytosolic proteins that primarily recognize bacterial components [64] as well as viral DNA and RNA in mammalian species. Members of the avian NLR family are not completely recognized and the role of this family in viral immunity is still unknown. NLRC5 is a member of the NLR family that is expressed in most cells. The inhibitory effects of NLRC5 on inflammatory pathways in the chicken are well documented. It is suggested that chicken NLRC5 is involved in the host antimicrobial immune response [42].

5. Type I, II, and III interferon responses

IFNs are classified into three categories (type I, type II, and type III IFNs). All IFNs signal through the Janus kinases (JAK)/ signal transducer and activator of transcription proteins (STAT) pathway which leads to the transcription of ISGs. However, cells responsible for the production of different IFNs and receptor specificity are different among IFNs. The swift induction of type I and type III IFN is essential for host immunity against viral infection [42].

Chicken type I interferons (IFNs) are essential in defending the host against viral infections. IFNs and interferon-stimulated genes (ISGs) can inhibit viral replication by preventing the entry of the virus into the host cells, abolishing translation processes, attaching to viral RNA, sequestering viral proteins and regulating host antiviral responses [69]. Following the activation of host PRRs, type I interferons (IFN-α and IFN-β) are produced which leads to the expression of ISGs [89].

However, viruses have their own properties to break, evade, or inhibit innate responses. For example, the NS1 protein in the influenza virus is able to shut down antiviral responses in host cells [90]. In fact, the NS1 protein of the influenza virus interferes with the induction or the production of IFNs which increases the pathogenicity of the influenza virus [91]. A previous study in chickens highlighted the role of the C-terminal domain of NS1 in the reduction of antiviral responses [92]. C-terminal domain of NS1 is able to interact with cellular proteins, including CPSF 30 (cleavage and polyadenylation specificity factor 30-kDa subunit) and poly(A)-binding protein II which results in the inhibition of the 3′-end processing of cellular pre-mRNAs and subsequently type I IFNs and ISGs [93]. Li et al. proved the critical role of NS1 protein in the pathogenicity of AIV in chickens by interfering with interferon induction in chickens. The amino acid residue of NS1 of AIVs at position 149 determines the potency of the virus to interfere with IFNs [94].

IBV induces a delayed activation of the IFN responses and is resistant to the antiviral state induced by type I IFNs. It was shown that viral accessory protein 3a is involved in this resistance and that knocking this protein out hampers the resistance of the virus to the IFN response. IBV engages various strategies to counteract type I IFN response, highlighting the importance of induced antiviral responses [95,96]. IBV inhibits IFN-mediated activation of antiviral genes through inhibition of STAT1 phosphorylation and subsequent nuclear translocation in a time-dependent manner during the late stages of infection [95,96].

ISGs are responsible for modulation of host cell metabolism and interaction with viral components to suppress virus replication [97]. In chickens, influenza virus infection upregulates type I IFNs [98]. IFNs produced by virus-infected cells increase the synthesis of Mx proteins, which accumulate in cells treated with IFN-I or infected with influenza viruses [99]. The antiviral activity of Mx proteins is well-explained in ISGs section. Studies have shown that antiviral activities of chicken IFN-α are not limited to *in vitro* systems since *in ovo* and *in vivo* inhibition of influenza virus (H9N2) replication were characterized [100]. Chicken IFN-α (chIFN-α) has been administrated orally as an antiviral and therapeutic agent in chickens against AIV. Oral administration of chIFN-α results in the induction of ISGs, including 2′,5′-OAS and Mx1 in the trachea and subsequent limitation of AIV replication and shedding in treated and infected chickens [101].

The type II interferon, IFN-ɣ, is a proinflammatory cytokine that has an essential role in the activation of host defense against intracellular pathogens and is a hallmark cytokine of T helper type 1 (Th1) cells. The biological activity of chicken IFN- ɣ is similar to its mammalian counterpart by inducing MHC class I and class II presentation. IFN-ɣ also tightly regulates the production of nitric oxide, an important cellular inhibitory mechanism against viral infections. In addition, chIFN-ɣ displays antiviral activities *in vitro* [100]. The direct antiviral activity of IFN-ɣ against AIV or IBV in chickens has not been characterized, but IFN-ɣ induces nitric oxide production, macrophage cell surface markers and up-regulation of some ISGs such as 2′-5′ OAS. Therefore, it is possible that IFN-ɣ through the initiation of RNase L pathway, indirectly, interferes with the replication of AIV or IBV in host cells [1,50,55].

IFN-λ is the only identified type III interferon, which has common upstream regulatory elements with type I interferons. This interferon is produced in many cells similar to type I interferons and triggers many of the same ISGs [97]. Chicken IFN-λ displayed similar viral inhibitory activity against the influenza virus (PR8) to that observed for the type I chicken IFNs. In addition, the antiviral properties of chicken IFN-λ are similar to those in humans [102]. Previous studies have shown that the intensity of interferon responses depends on the AIV subtype and LPAI infections in chickens induce lower interferon responses than HPAI infections [103,104]. Recently, RNA-sequencing of human lung A549 cell line infected with IBV showed an upregulation of type III IFN mRNA, including IFN-λ1, IFN-λ2, and IFN-λ3. Several antiviral ISGs antiviral effectors in the innate immune system, including OAS proteins, MX proteins, IFITM proteins, and viperin were also highly upregulated [105].

6. Interferon-stimulated genes (ISGs)

Following the induction of type I IFNs in infected tissues or activated with PAMPs, ISGs are expressed to set an antiviral status. ISGs products are necessary for protection against viral infections. Some ISGs that have been experimentally characterized in chickens, including Viperin (RSAD2), interferon-induced proteins with-tetratricopeptide repeats (IFIT)5, PKR (EIF2AK2), Chemokine (C-C motif) ligand (CCL)19, Mx, and IFITM3 and 2′-5′ OAS [97].

Viperin is an antiviral ISG with enzymatic activity. It prevents releasing of newly-synthesized influenza virion by perturbing lipid rafts during virus assembly [106–108]. Chicken viperin is activated by several TLR ligands and AIV infection. Previous studies in chickens demonstrated the significant expression of viperin in different cells, including macrophages, tracheal cells, and cecum following TLR ligands treatments [47,50]. Chickens possess potentially functional viperin, but

it is still unknown whether they can inhibit viral replication or not [97]. In Burggraaf et al. study, the levels of chicken viperin were measured in the lung and spleen after experimental infection with H5N1 (A/Muscovy duck/Vietnam/453/2004) AIV. In this study, significant up-regulation of viperin was detected in the lung and spleen of infected chickens [109].

IFITs are a group of IFN-effectors that directly engage with 5′-ppp viral RNA [110,111]. IFIT5 is the only IFIT gene that has been identified in chickens [112]. Rohaim et al. suggested that the stable expression of chIFIT5 alone is insufficient to provide protection against HPAIV, but improves the clinical symptoms [113]. Recently, it is demonstrated that the chIFIT5 gene is responsive to both type I IFNs (IFN-α and IFN-β) and AIV infection. In addition, chIFIT5 can specifically antagonize with negative-sense viral RNA structures carrying 5′-ppp portion. A previous study in chickens demonstrated the interaction between AIV and IFIT5 and subsequent reduction of AIV replication by using a lentivirus-mediated IFIT5-stable chicken fibroblast model [110]. Furthermore, the kinetics of AIV replication in transgenic chicken embryos (expressing chIFIT5 protein or knocked-down for endogenous chIFIT5 gene) showed the potential antiviral activity of chIFIT5 against AIV *in ovo* [110]. Infection of different chicken tissues (liver, kidney, spleen, beak, trachea, lungs, and duodenum) with H9N2 avian influenza virus strain showed higher levels of chIFIT5 expression and it seems that AIV infection positively regulates the transcriptional dynamics of chIFIT5 in chickens [110].

IFITM family is known to have a critical role in limiting AIV infection in chickens [114,115]. Clustering of gene expression profiles in chickens suggests that IFITM1 and 2 have an antiviral response and IFITM3 may act prior to viral membrane fusion and thus blocks viral entrance [114]. In physiological status, IFIRM5 is the main expressed IFITM in different embryonic and adult organs in chickens, except for the colon in which IFITM1 and 3 are the main expressed IFITMs. However, a previous study in chickens demonstrated the significant expression of IFITM5 in CAM of embryonated eggs following the treatment with TLR2, 4 and 7 ligands. The expression of IFITM3 is increased following IBV infection in chickens [116]. The expression of IFITM3 is significantly increased in the lung of infected chickens with AIV [117]. IFITM3 is a viral restriction factor, which targets influenza A virus entry process in humans by blocking complete virus envelope fusion with cell endosome membranes [115,118]. It has been suggested that the chicken IFITM3 protein protect host cells from infection with AIV to a similar level as its human orthologue and *in vitro* experiments showed the anti-influenza activity of IFITM3 in chicken cells [114]. Barjesteh et al. *in vivo* study demonstrated the up-regulation of IFITM5 in the chicken trachea cells following CpG ODN and Pam3CSK4 treatments [49]. In addition, IFITM3 is up-regulated in trachea after CpG treatment of chickens *in vivo*, which may be the reason for the largest reduction in oral AIV shedding by CpG 1826 since IFITM3 inhibits the replication of AIV in host cells by preventing viral uncoating [47,115,119]. Furthermore, it has been shown that HPAI H5N1 virus can induce excessive expression of IFITM5 in lung tissues of chickens. Conversely, the IFITM5 gene is down-regulated in the lung tissues following infection of chickens with LPAIV H9N2. It is suggested that this dysregulation of IFITM5 gene may contribute to the severity and the outcome of the influenza infection in chickens [120].

PKR is a double-stranded (ds) RNA-dependent serine/threonine-protein kinase with antiviral, antiproliferative, and pro-apoptotic activities [97]. Binding of dsRNA in the cytoplasm activates the kinase activity of PKR. Then it interferes with viral replication and cell proliferation via inhibiting protein translation [121,122]. It has been revealed that PKR-knockout mice are highly susceptible to influenza A virus infection. Chicken PKR has antiviral activities against RNA viruses [97,123]. However, induced PKR following H5N1AIV infection fails to produce an effective antiviral response against HPAIV H5N1 [124]. PKR expression is increased in chickens in different cells and tissues, including trachea, cecum, lung, splenocytes, and macrophages following TLR2, 4, 7 and 21 ligand treatment [47,49,50].

GTP-binding protein Mx is a well-known and critical ISG against the influenza virus in mammals [97]. While there are two Mx genes in humans (MxA and MxB) and mice (Mx1 and Mx2), a single polymorphic Mx gene has been recognized in chickens [125,126]. Cytoplasmic human Mx interacts directly with influenza nucleoproteins (NP), while nuclear MxA blocks viral RNA transcription [127]. Mx is principally induced by type I IFN [124]. The study of Schusser et al. on chicken Mx revealed that this protein lacks GTPase activity [128]. Previously, it was suggested that GTP hydrolysis is essential for Mx antiviral function, but recent studies in chickens have shown that Mx protein is not able to induce protection against influenza and Mx lacks GTPase activity [100,129,130]. A weak host immune response is observed in chickens infected with HPAI H5N1 strain of AIV, in spite of the induction of IL-6, Mx, and PKR. It is suggested that the induction of the antiviral proteins PKR and Mx without an increase in type I IFNs fails to produce effective antiviral responses against HPAIVs [124].

2′-5′oligoadenylate synthetase (OAS) is an ISG protein in chickens. Activation of the classical OAS/RNase L pathway results in RNA degradation of both viral and cellular RNA, including ribosomal RNA within infected cells. Subsequently, it inhibits protein translation in these cells, which leads to effective prevention of viral genome replication [131,132]. The expression of OAS is increased following viral infections, including IBV [96]. In addition, several studies have shown the significant mRNA expression of OAS gene following TLR ligand treatments in chickens. Previous studies demonstrated that TLR2, 4 and 21 ligands Pam3CSK4, LPS, and R848, respectively, induced the expression of some ISGs, including OAS in the CAM, trachea and cecal tonsils. In contrast, TRL21 ligand CpG ODN 1826 does not induce 2′-5′ OAS in the CAM [63]. Another study indicated that LPS and Pam3CSK4 significantly induce the expression of OAS in the chicken trachea and cecal tonsils. The findings of the Barjesteh et al. suggested that TLR ligands can interfere with the replication of AIV, which may be correlated with the expression of OAS [63]. In addition, it is evident that IFN-ɣ induces nitric oxide production, macrophage cell surface markers and up-regulation of some ISGs such as 2′-5′ OAS. Therefore, it might be that IFN-ɣ through the initiation of RNase L pathway, indirectly, interferes with the replication of AIV in macrophages.

7. Conclusion

The host-pathogen interactions of the chicken innate system and viruses are multifactorial and complex. The combination of the virus replication in host tissues, and activation of the PRRs and their downstream IFNs and pro-inflammatory cytokines suggests that the chicken innate responses play a key role in the control of viral infections. The host innate responses either clear invading viruses or allows the adaptive immune system to establish an effective antiviral response. Although our knowledge about chicken innate responses against respiratory viral infections has significantly expanded in the last decade, a better understanding of cross-talk between different host cells and involved underlying mechanisms is required. Understanding the molecular mechanisms involved in the interaction between different host cells is crucial for further improvements in strategies.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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