REVIEW

The pros and cons of cytokines for fowl adenovirus serotype 4 infection

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Abstract

Hepatitis-hydropericardium syndrome (HHS), caused by fowl adenovirus serotype 4 (FAdV-4), has spread on chicken farms worldwide, causing huge economic losses. Currently, the exact mechanism of pathogenesis of FAdV-4 remains unknown. Despite the severe inflammatory damage observed in chickens infected with pathogenic FAdV-4, few studies have focused on the host immune system-virus interactions and cytokine secretion. Host immunity acts as one of the most robust defense mechanisms against infection by pathogens, and cytokines are important in their elimination. However, excessive inflammatory cytokine secretion could contribute to the pathogenesis of FAdV-4. Understanding of the roles of cytokines produced during FAdV-4 infection is important for the study of pathogenicity and for developing strategies to control FAdV-4. Several previous studies have addressed the immune responses to FAdV-4 infection, but there has not been a systematic review of this work. The present review provides a detailed summary of the current findings on cytokine production induced by FAdV-4 infection to accelerate our understanding of FAdV-4 pathogenesis.

Introduction

Fowl adenoviruses (FAdVs) belong to the genus *Aviadenovirus* of the family *Adenoviridae* and are subdivided into five species, *Fowl adenovirus A* to *E*, and 12 serotypes, including FAdV-1 to 7, 8a and 8b, and 9 to 11 [1]. Fowl adenovirus serotype 4 (FAdV-4) of the species *Fowl adenovirus C* is the etiological agent of hepatitis-hydropericardium syndrome (HHS) [2]. Since the first case was reported in 1987 in Pakistan, HHS has become prevalent in Asia, Australia, and South and North America, causing huge economic losses to the global poultry industry [3]. Since May 2015, outbreaks of HHS caused by a novel genotype of FAdV-4 have been reported in China [4].

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FAdV-4 is a double-stranded, non-enveloped, icosahedral-shaped DNA virus with an approximately 45-kb genome encoding 11 structural proteins and 32 non-structural proteins. The fiber-1, fiber-2, hexon, and penton base structures form the viral capsid, with fibers connecting to the penton base [5]. FAdV-4 infection mainly affects 3-week to 6-week-old broiler chickens, causing up to 80% mortality. The onset of the disease is characterized by lethargy, diarrhea, anemia, nephritis, ruffled feathers, and weight loss. Necropsy findings include swollen liver with focal necroses, petechial hemorrhages, enlarged pericardial sacs filled with transparent and straw-color fluids, and swollen kidneys [2]. FAdV-4 infection also causes the depletion of B and T cells in lymphoid organs such as the thymus and spleen of infected chickens [6]. Coinfections of FAdV-4 with other immunosuppressive pathogens such as chicken infectious anemia virus (CIAV), Marek's disease virus (MDV), infectious bursal disease virus (IBDV), avian orthoreovirus (ARV), and Newcastle disease virus (NDV) usually cause more- severe outcomes [7–13].

The immune system is one of the most important and robust guards against viral infection. Proper immune responses effectively inhibit virus replication and limit viral propagation. The interaction of the virus with the host immune system triggers cascades of intracellular signaling events and the engagement of specific cellular receptors and

cytokines. Cytokines produced by the immune responses have important local and systemic effects that contribute to both innate and adaptive immunity to viral infection. However, an excessive immune response to infection is sometimes harmful to the host. Fever, malaise, and inflammatory damage are often observed when a large quantity of cytokines is released in an uncontrolled manner. FAdV-4 replicates extensively in the liver, heart, spleen, lungs, and kidneys of infected chickens. Increased expression of inflammatory cytokines is observed in the principal organs targeted by the virus, but little is known about the function of each cytokine. Although the mechanism of FAdV-4 pathogenesis has not been completely elucidated, understanding the roles of cytokines is undoubtedly crucial. Studies on the immune responses triggered by FAdV-4 have been limited in scope and focused on a few specific cytokines. Therefore, the present review starts with a discussion of the recognition of FAdV-4 by the immune system and summarizes what is currently known about the expression of various cytokines in response to FAdV-4 infection in chickens. Gaps requiring further investigation will be highlighted in order to identify important research topics. In addition, our unpublished data on cytokine expression in the organs targeted by FAdV-4 will be discussed for each cytokine in comparison to previous studies.

Recognition of FAdV-4 by the immune system

The initial recognition of the invading pathogen by the innate immune system of chickens is accomplished by different types of pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). These immune sensors act as the first line of defense and help in the maintenance of immune homeostasis. Once they are activated, multiple downstream signaling regulators are triggered, including nuclear factor-κB (NF-κB), mitogen-activated protein kinases (MAPKs), and interferon regulatory factors (IRFs), which further facilitates the expression of inflammatory cytokines, chemokines, and interferons [14, 15].

TLRs

TLRs are membrane-bound PRRs that recognize microbial-pathogen-associated molecules, including lipopolysaccharides, proteins, and viral nucleic acids. TLRs are composed of an extracellular N-terminal domain responsible for detection of pathogen-associated molecular patterns (PAMPs), a transmembrane domain, and an intracellular Toll-interleukin 1 (IL-1) receptor (TIR) domain, which mediates downstream

signal transduction [15]. Currently, 10 chicken TLRs have been characterized, including TLR1La, TLR1Lb, TLR2a, TLR2b, and TLR3-5, 7, 15, and 21. Chicken TLR2a, 2b, 4, 5, and 7 are orthologous to the human and mouse TLRs, while chTLR1La, 1Lb, 15, and 21 are unique to birds. TLRs are important for the production of cytokines and the subsequent antiviral response, especially the secretion of type I interferons (IFN) [16]. TLR2 recognizes a wide range of microbes, including Gram-positive and Gram-negative bacteria, fungi, viruses, and parasites. TLR3 plays an important role in defending against virus invasion by upregulating the expression of antiviral type I IFN. TLR4 recognizes lipopolysaccharides (LPSs), and TLR5 senses oxidative stress to induce a pro-inflammatory response [17]. TLR7, a highly conserved functional protein residing in endosomes, recognizes ssRNA. TLR15 was first identified as being upregulated after infection of chickens with salmonella. It is highly expressed in the marrow and the bursa of Fabricius (BF) and expressed at a low level in the tongue, liver, spleen, cecum, and small intestine of healthy chickens. TLR15 is important in the immune response against bacterial and viral infections [16, 18]. Chicken TLR21 acts as a functional homologue to mammalian TLR9 in the recognition of CpG oligodeoxynucleotides [19].

Elevated expression of TLRs in chickens infected with FAdV-4 has been observed in several studies (Table 1). After intraocular inoculation of virulent FAdV-4 strain HN/151025 in 60-day-old chickens, Zhao et al. observed that the expression of several TLRs was significantly upregulated in the infected spleens, including TLR1 at 7 dpi, TLR4 at 36 hpi, and TLR21 at 36 hpi and 7 dpi, when compared to a control group [20]. Notably, the expression of TLR2, 3, 5, 7, and 15 showed little statistical difference between infected and uninfected spleens. In the infected BF, the expression of TLR4 increased at 36 hpi and 7 dpi. In addition, the expression of TLR1-3, 5, 7, 15, and 21 decreased at 36 hpi in the BF, but they increased significantly later at different time points: TLR1, 3, 5, 7, 15, and 21 increased at 7 dpi and TLR1, 5, 7, and 15 increased at 20 dpi. Upregulation of TLRs was also observed in the bone marrow, heart, liver, and cecal tonsils. Strong and positive correlations were observed between the FAdV-4 viral load and expression levels of TLR2 and 7 in the spleen, of TLR1, TLR2, TLR7, and TLR15 in the BF, and of TLR2, TLR3, TLR7, and TLR15 in the liver of the infected chickens [20]. Meng et al. challenged 21-day-old chickens intramuscularly (IM) with FAdV-4 strain SDJN0105 and observed a 10-fold and 14-fold increase of TLR3 and an 8-fold and 16-fold increase of TLR7 in the liver and spleen, respectively, at 2 dpi [21]. Li et al. infected 21-day-old chickens with FAdV-4 strain SD0828 through intramuscular administration and observed that the expression levels of TLR3 and TLR7 increased significantly at 1 dpi but returned to baseline at 2 dpi in the liver

Table 1 Changes in the expression of immune sensors in different organs of chickens infected with FAdV-4

Immune sensor	Strain	Route	Organ			Ref
			Liver	Spleen	Bursa of Fabricius	
TLR1	HN/151025	IO		↑ (7d)	↓ (36h) ↑ (7&20d)	[20]
TLR2	HN/151025	IO		--	↓ (36h)	[20]
TLR3	HN/151025	IO		--	↓ (36h) ↑ (7d)	[20]
	SDJN0105	IM	↑ (2d)	↑ (2d)		[21]
	SD0828	IM	↑ (1d)	↑ (1d)		[22]
		Oral	--	--		
TLR4	HN/151025	IO		↑ (36h)	↑ (36h) ↑ (7d)	[20]
TLR5	HN/151025	IO		--	↓ (36h) ↑ (7&20d)	[20]
TLR7	HN/151025	IO		--	↓ (36h) ↑ (7&20d)	[20]
	SDJN0105	IM	↑ (2d)	↑ (2d)		[21]
	SD0828	IM	↑ (1d)	↑ (1d)		[22]
		Oral	--	--		
TLR15	HN/151025	IO		--	↓ (36h) ↑ (7&20d)	[20]
TLR21	HN/151025	IO		↑ (36h&7d)	↓ (36h) ↑ (7d)	[20]
NLRP3	CH/HNJZ/2015	IM	↑ (2&3d)	↑ (1d)	↑ (3d)	U/D
MDA5	SDJN0105	IM	↑ (2&3d)	↑ (1, 2&3d)		[21]
	SD0828	IM	↑ (1 to 2d)	↑ (1 to 2d)		[22]
		Oral	-↑	-↑		

IM, intramuscular; IO, intraocular; ↑, significant upregulation; ↓, significant downregulation; h, hours postinfection; d, days postinfection; --, no significant change; Ref, reference; U/D, unpublished data

and spleen. However, the expression of TLR3 and TLR7 in the oral administration group remained around baseline from 1 dpi to 5 dpi [22].

NLRP3 inflammasomes

In addition to TLRs, our unpublished data indicate that nod-like receptor family pyrin domain containing 3 (NLRP3) inflammasomes also play a major role in the immune response to FAdV-4 infection. NLRP3 inflammasomes are composed of a sensor protein (NLRP3), an adaptor protein (ASC), and a downstream effector (pro-caspase-1) [23]. Similar to TLRs, NLRP3 inflammasomes serve as sensors of the innate immune system in response to invading PAMPs. Upon activation, NLRP3 triggers the conversion of pro-caspase 1 to active caspase 1, which in turn cleaves pro-IL-18 and pro-IL-1β to generate pro-inflammatory cytokines IL-18 and IL-1β [24]. Unlike chicken TLRs, the role of chicken NLRP3 inflammasomes in viral infection is rarely studied. According to our data, NLRP3 is significantly upregulated in the spleen at 1 dpi, in the heart, kidney, and BF at 3 dpi, and in the liver at 2 and 3 dpi of 3-week-old SPF chickens infected intramuscularly with the pathogenic FAdV-4 strain CH/HNJZ/2015 (Table 1). In addition, we found that NLRP3 is activated in the chicken macrophage cell line HD11 infected with CH/HNJZ/2015, and the expression of IL-1β is significantly upregulated. This provides evidence that NLRP3 inflammasomes play a role in the immune response

to FAdV-4 infection, but the mechanism by which chicken NLRP3 inflammasomes are activated during FAdV-4 infection needs further elucidation.

MDA5

Unlike ducks and geese, chickens naturally lack retinoic-acid-inducible gene I (RIG-I)-like receptors, but melanoma differentiation-associated gene 5 (MDA5) compensates for this deficiency by recognizing both DNA and RNA viruses. MDA5 is a key cytosolic PRR that detects viral infection and regulates the expression of interferons [16]. Several studies have indicated that MDA5 recognizes FAdV-4 infection (Table 1). Meng et al. observed significant upregulation of MDA5 at 2 dpi in the heart, spleen, and liver of chickens infected intramuscularly with SDJN0105, and the highest expression was observed in the spleen [21]. Li et al. reported that the expression of MDA5 showed sustained upregulation from 1 to 2 dpi in the liver and spleen of chickens infected intramuscularly with SD0828, and a similar but smaller increase was observed in chickens infected via the oral route [22]. A more recent study performed by Li et al. also found a 19.5-fold increase in MDA5 in primary chicken embryo kidney cells infected with AH-FAdV-4 [25].

In summary, FAdV-4 infection is sensed by receptors of the innate immune system including TLRs, NLRP3 inflammasomes, and MDA5, which in turn regulate the production of inflammatory cytokines. Multiple TLRs, including

TLR1-4, 7, 15, and 21, work synergistically in response to FAdV-4 infection. This suggests that different signaling pathways are utilized against FAdV-4, and further studies are needed to identify the signaling pathways and immune sensors that are involved.

Expression of inflammatory cytokines of innate immunity

The interleukin family

The interleukin (IL) family is composed of a large group of pro- or anti-inflammatory cytokines that play a variety of roles in the immune response to viral infection, including regulation of the maturation and proliferation of immune cells, immunomodulation, and inflammation. There is accumulating evidence that interleukins contribute to FAdV-4-induced inflammatory injury.

The IL-1 family (IL-1β and IL-18)

The IL-1 family has 11 members (IL-1α, IL-1β, IL-1RA, IL-18, IL-33, IL-36Ra, IL-36α, IL-36β, IL-36γ, IL-37, and IL-38) [26], among which IL-1β and IL-18 are the best-studied cytokines in FAdV-4 infection (Table 2). Meng et al., who infected chickens with the pathogenic FAdV-4 strain SDJN0105 via the IM route, reported that the levels of IL-1β mRNA increased significantly in the heart, spleen, and liver from 1 to 3 dpi. A 90-fold increase was observed in the spleen at 2 dpi [21]. Zhao et al. observed a significant increase in the expression level of IL-1β at 36 hpi in the spleen and of IL-1β and IL-18 at 7 dpi in the BF [20]. Strong and positive correlations were observed between the viral load and the expression level of IL-1β in the infected spleen, liver, bone marrow, and BF and the level of IL-18 in the spleen and liver. Li et al. observed a 46-fold increase of IL-1β expression at 1 dpi in the spleen of chickens infected with FAdV-4 SD0828 by the IM route [22]. Niu et al. infected 7-day-old SPF chickens with FAdV-4 strain SDDM-4/15 via the subcutaneous route and observed a 33-fold increase of IL-1β expression in the liver at 3 and 4 dpi. Notably, the expression of IL-1β was also significantly upregulated in LMH cells infected with SDDM-4/15 [27]. In another study performed by Niu et al., the expression of IL-1β significantly increased in the heart of 7-day-old SPF chickens infected with FAdV-4 strain SDDM-4/15 at 4 dpi [28].

Grgić et al. evaluated changes in cytokine expression associated with infection with the non-virulent FAdV-4 strain ON1 [29]. Chickens inoculated with this strain showed no significant difference in the expression of IL-18 in the liver and cecal tonsils of infected birds compared to that in the control group. Interestingly, significant downregulation of IL-18 was observed in the spleen of infected chickens at 10 dpi [29].

We have conducted animal experiments to evaluate the expression of cytokines induced by pathogenic and non-pathogenic FAdV-4 strains. SPF chickens were infected intramuscularly with either the pathogenic strain CH/HNJZ/2015 or the non-pathogenic strain ON1 and euthanized at 24 and 48 hours postinfection. Organs targeted by the virus, including heart, liver, spleen, lungs, kidneys, cecal tonsils, BF, duodenum, proventriculus, and pancreas, were collected for qRT-PCR examination. The results indicated significant upregulation of IL-1β in the BF at 24 hpi and in the heart, liver, spleen, lungs, cecal tonsils, and BF at 48 hpi in chickens infected with CH/HNJZ/2015 compared to the control group. The expression of IL-1β in chickens infected with ON1 increased significantly in the liver, lungs, and BF at 24 hpi and in the spleen and BF at 48 hpi compared to the control group. The mRNA expression level of IL-18 increased significantly in the heart and lungs at 24 hpi and in the heart, spleen, lungs, cecal tonsils, duodenum, pancreas, and proventriculus at 48 hpi in chickens infected with CH/HNJZ/2015 compared to the control group. In contrast, the expression level of IL-18 increased significantly only in the lungs of chickens infected with ON1 at 24 hpi. In addition, the expression of IL-1β in all organs except the BF was significantly higher in CH/HNJZ/2015-infected chickens than in ON1-infected chickens; the expression of IL-18 was significantly higher in the heart, spleen, lungs, cecal tonsils, duodenum, proventriculus, pancreas, and thymus of chickens infected with CH/HNJZ/2015 than in chickens infected with ON1.

IL-1β is produced as a biologically inert pro-peptide that requires proteolytic processing for activation. The N-terminal region of pro-IL-1β is removed by cleavage by caspase 1, neutrophil serine proteases, or mast-cell-derived serine proteases upon inflammasome activation. IL-1β binds to interleukin 1 receptor 1 (IL-1R1) to form a ternary complex that then recruits intracellular signaling molecules, including myeloid differentiation factor 88 (MyD88), IL-1R-associated kinase (IRAK), and TNF receptor-associated factor 6 (TRAF6) to activate nuclear factor-κB (NF-κB) as well as p38, c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and mitogen-activated protein kinases (MAPKs) [30, 31]. IL-1β promotes the differentiation of monocytes to conventional dendritic cells and M1-like macrophages, which participate in pathogen eradication. IL-1 also induces the upregulation of adhesion receptors on immune and endothelial cells for infiltration of leukocytes to sites of infection [32]. However, the concept of IL-1β being a beneficial immune regulator has been challenged, as IL-1β cytotoxicity has been implicated in a broadening list of diseases, especially in liver injury,

Table 2 Changes in the expression of inflammatory cytokines in different organs of chickens infected with FAdV-4

Cytokine	Strain	Route	Heart	Liver	Spleen	Cecal tonsil	Bursa of Fabricius	Thymus	Ref
IL-1β	SDJN0105	IM		↑ (2d)	↑ (2d)				[21]
	SD0828	IM			↑ (1d)				[22]
		Oral			-↑				
	SDDM-4/15	SC		↑ (3&4d)					[27]
			↑ (4d)						[28]
	HN/151025	IO			↑ (36h)		↓ (36h) ↑ (7d)		[20]
	CH/HNJZ/2015	IM	↑ (48h)	↑ (48h)	↑ (48h)	↑ (48h)	↑ (24h&48h)	--	U/D
	ON1		--	↑ (24h)	↑ (48h)	--	↑ (24h&48h)	--	
IL-18	HN/151025	IO					↑ (7d)		[20]
	ON1	IM		--	↓ (10d)	--			[29]
	CH/HNJZ/2015		↑ (24&48h)	--	↑ (48h)	↑ (48h)	--	--	U/D
	ON1		--	--	--	--	--	--	
IL-6	SDJN0105	IM			↑ (2d)				[21]
	SD0828	IM			↑ (1d)				[22]
		Oral			-↑				
	SDDM-4/15	SC		↑ (2&3d)					[27]
					↑ (3&4d)		↑ (3d)	↑ (3d)	[46]
			↑ (3d)						[28]
	HN/151025	IO			↑ (36h)		↓ (36h) ↑ (7&20d)		[20]
	CH/HNJZ/2015	IM	↑ (48h)	--	--	↑ (24&48h)	↑ (48h)	--	U/D
	ON1		--	--	--	--	--	--	
IL-8	SDJN0105	IM	↑ (2d)	↑ (2d)	↑ (2d)				[21]
	SD0828	IM		↑ (1d)	↑ (1d)				[22]
		Oral		-↑	-↑				
	SDDM-4/15	SC		↑ (2 to 5d)	↑ (2 to 5d)				[27]
					↑ (1, 3, 4, 5d)		↑ (1, 3, 4d)	↑ (3d)	[46]
			↑ (2 to 3d)						[28]
	CH/HNJZ/2015	IM	↑ (24&48h)	↑ (48h)	--	↑ (48h)	--	--	U/D
	ON1		--	--	--	--	--	--	
IL-10	ON1	IM		↑ (3d)					[29]
	SX17			↑ (5d)				↑ (5d)	[68]
	CH/HNJZ/2015		↑ (24&48h)	↑ (48h)	--	--	↑ (24&48h)	↑ (24&48h)	U/D
	ON1		--	--	--	--	↑ (24h)	↑ (24&48h)	
TNF-α	SDJN0105	IM	↑ (2d)	↑ (2d)	↑ (2d)				[21]
	SDDM-4/15	SC		↑ (2 to 5d)					[27]
					↑ (1, 3, 4d)		↑ (3d)	↑ (3d)	[46]
			↑ (2 to 3d)						[28]
	CH/HNJZ/2015	IM	↑ (48h)	--	--	↑ (48h)	--	↑ (48h)	U/D
	ON1		--	--	--	↑ (48h)	--	--	

IM, intramuscular; IO, intraocular; SC, subcutaneous; ↑, significant upregulation; ↓, significant downregulation; h, hours postinfection; d, days postinfection; --, no significant change; -↑, smaller increase; Ref, reference; U/D, unpublished data

autoinflammatory diseases, and chronic, debilitating syndromes [33, 34]. Previous research has indicated that inhibiting the generation of inflammasome-dependent IL-1β limits acute toxic liver injury in mice [35]. IL-1β has been demonstrated to play an important role in other poultry diseases as well. Infectious bronchitis virus (IBV) infection induces upregulation of IL-1β expression in the trachea and lungs of chickens [36]. Anti-IL-1β neutralizing antibody treatment was found to decrease the mortality rate in chickens after infection with virulent NDV [37]. The multifaceted role of IL-1β in the pathogenicity of FAdV-4 is vague and requires in-depth elucidation. According to our unpublished data, the

mortality rate in chickens infected with recombinant CH/HNJZ/2015 expressing chicken IL-1β was much lower than when wild-type CH/HNJZ/2015 was used. This implies that active expression of IL-1β at an early stage of infection inhibited FAdV-4 replication and reduced the pathogenicity of CH/HNJZ/2015.

IL-18 is known as the interferon gamma (IFN-γ)-inducing factor and is structurally similar to IL-1β. IL-18 receptors are present on a wide range of cells, including immune cells such as T cells, natural killer cells, B cells, and macrophages, and non-immune cells, such as endothelial and epithelial cells. IL-18 transduction leads to the production of IFN-γ from T, B, and NK cells [38], promotes nitric oxide (NO) secretion and Th1 immunity, and activates T cell proliferation [39]. Prokaryotically expressed IL-18 protein significantly increased IFN-γ secretion in chicken splenocytes. Co-administration of IL-18 protein with cell-cultured Newcastle disease vaccine or inactivated AI H9N2 vaccine led to an effectively enhanced cell-mediated and humoral immunity [40–42]. Similarly, co-expression of IL-18 with infectious laryngotracheitis virus DNA vaccine or infectious bursal disease virus (IBDV) DNA vaccine provided better protection against virus challenge [43, 44], demonstrating the potency of IL-18 in preventing virus propagation. Pre-treating chickens with prokaryotically expressed IL-18 and/or IFN-γ protein effectively inhibited the proliferation of IBDV [45]. Investigation of the potential value of IL-18 as a vaccine adjuvant would be worthwhile for developing strategies for a new generation of FAdV-4 vaccines. Although most of previous studies have focused on IL-1β, IL-18 is worthy of further study, as our results indicate that the expression of IL-18 differs in chickens infected with FAdV-4 strains with different pathogenicity.

IL-6

The expression of IL-6 was found to increase significantly in multiple organs of chickens infected with virulent-FAdV-4 (Table 2). Infection with FAdV-4 strain SDJN0105 led to 954.3-fold increase of IL-6 in the spleen at 2 dpi [21]. Similarly, a 909.51-fold increase in IL-6 was observed in the spleen of chickens infected with FAdV-4 strain SD0828 at 1 dpi [22]. A significant increase in IL-6 expression was also detected in FAdV-4 SDDM-4/15-infected liver (24.21-fold and 47.45-fold at 2 and 3 dpi) [27], heart (25.83-fold at 3 dpi) [28], and LMH cells at 12, 24, and 36 hpi [27]. Niu et al. inoculated chickens with FAdV-4 SDDM-4/15 and observed significant upregulation of IL-6 in the spleen (33.12- and 14.75-fold at 3 and 4 dpi), thymus (9.8-fold at 3 dpi), and BF (18.43-fold at 3 dpi) [46]. The expression level of IL-6 in chickens infected with FAdV-4HN/151025 increased significantly in the spleen at 36 hpi but did not increase in the BF until later time points (7 and 20 dpi). A strong and positive correlation was observed between the viral load and the expression level of IL-6 in the BF [20].

In our research, the expression level of IL-6 increased significantly in the lungs, cecal tonsils, and pancreas at 24 hpi and in heart, lungs, cecal tonsils, duodenum, BF, and proventriculus at 48 hpi in chickens infected with CH/HNJZ/2015 compared to the control group. In chickens infected with ON1, IL-6 was upregulated in the lungs and pancreas at 24 hpi and in the heart, duodenum, and proventriculus at 48 hpi compared to the control group. The overall expression of IL-6 was higher in chickens infected with CH/HNJZ/2015 than in those infected with ON1.

The multipotent cytokine IL-6 plays a role in regulating immune responses, acute-phase reactions, and hematopoiesis. It exerts effects on a broad range of cells, making it both proinflammatory and anti-inflammatory [47]. IL-6 binds to functional IL-6 receptors located on the cytoplasmic membrane to stimulate the Janus kinase (Jak) family of tyrosine kinases and activate the signal transducer and activator of transcription (STAT) protein, which translocates to the nucleus and regulates the expression of specific genes such as vascular endothelial growth factor (VEGF), which sometimes participates in the process of oncogenesis [48, 49]. Previous research has shown that avian leukosis virus subgroup J virus dysregulates IL-6 to promote tumorigenesis [49]. On the other hand, IL-6 activates B cells and regulates antibody production. Co-administration of expression plasmids encoding IL-6 and VP2-4-3 from vvIBDV conferred protection to 90% of chickens against vvIBDV challenge, whereas only 15% of chickens were protected when receiving the VP2-4-3 plasmid alone [50]. In the case of FAdV-4 infection, IL-6 plays a vital role, as its expression is generally higher in chickens infected with pathogenic FAdV-4. However, it remains unclear whether IL-6 is beneficial to chickens infected with FAdV-4.

IL-8

Proinflammatory cytokine IL-8 is secreted mainly by macrophages and endothelial cells and is known to mediate inflammation by recruiting and activating neutrophils to the site of infection [51]. Multiple studies have highlighted the importance of IL-8 in FAdV-4 infection (Table 2). Chickens inoculated with FAdV-4 SDJN0105 showed significant upregulation of IL-8 in the heart, liver, and spleen (217-fold) at 2 dpi [21]. Inoculation with FAdV-4 SD0828 via the IM route led to an increase in expression levels of IL-8 in the liver (99-fold) and spleen at 1 dpi, and inoculation via the oral route also led to an increase, but a smaller one [22]. Inoculation with FAdV-4 SDDM-4/15 led to a significant increase in IL-8 in the heart, liver, spleen, and thymus of infected chickens and in LMH cells [27, 28, 46]. More specifically, the expression levels of IL-8 increased by 616-fold

at 3 dpi in the thymus, 36.19-, 2124.06-, 1817.91-, and 170.07-fold at 1, 3, 4, and 5 dpi in the spleen and 136.32-, 617.63-, and 178.69-fold at 1, 3, and 4 dpi in the BF, respectively [46]. In contrast to infection with virulent FAdV-4, infection with ON1 did not result in a significant change in the expression level of IL-8 in the spleen, liver, or cecal tonsils of the infected chickens [29].

Our results showed that the expression level of IL-8 in chickens infected with CH/HNJZ/2015 increased significantly in the heart and lungs at 24 hpi and in the heart, liver, lungs, cecal tonsils, duodenum, and proventriculus at 48 hpi. In contrast, in chicken infected with ON1, it only increased significantly in the lungs at 24 hpi. It could be assumed that IL-8 was significantly upregulated in chickens infected with the pathogenic strain of FAdV-4 to participate in the pathogen removal process, especially in the liver and spleen, and it is not activated in chickens infected with non-pathogenic strains.

IL-10

Wu et al. infected 21-day-old SPF chickens with FAdV-4 isolate SX17 and found that the expression of IL-10 increased significantly in both the liver and thymus of the infected chickens at 5 dpi (11.81- and 11.71-fold, respectively). Inoculating chickens with the non-virulent FAdV-4 strain ON1 leads to significant upregulation of IL-10 in the liver at 3 dpi [29]. In our research, we found that the expression of IL-10 increased significantly in the heart, BF, proventriculus, and thymus at 24 hpi and in the heart, liver, lungs, BF, duodenum, proventriculus, pancreas, and thymus at 48 hpi of chickens infected with CH/HNJZ/2015, and in the lungs, BF, and thymus at 24 hpi and in the proventriculus and thymus at 48 hpi in chickens infected with ON1. The expression of IL-10 in CH/HNJZ/2015-infected lungs, duodenum, and pancreas was significantly higher than in ON1-infected organs at 48 hpi.

IL-10 is a pleiotropic anti-inflammatory cytokine that limits and terminates the immune response induced by viral infection and thus alleviates the inflammatory damage caused by the host immune system and restores homeostasis [52]. The significant upregulation of IL-10 found in FAdV-4-infected chickens might contribute to the anti-inflammatory activity in the primary target organ of FAdV-4. However, the exact cellular pathway that triggers the secretion of IL-10 by FAdV-4 is unclear, and there is less information about the exact function of IL-10 in FAdV-4-infected birds.

Tumor necrosis factor α (TNF-α)

TNF-α, which is secreted by macrophages in response to stresses, is a major proinflammatory cytokine that activates the NF-κB or MAPK pathway. It shows functional duality by its engagement in inflammation, tissue regeneration, and apoptosis [53]. Previous research indicated that TNF-α stimulates its own production by macrophages as well as the production of IL-1β and IL-6 [54]. The mRNA expression level of TNF-α has been shown to increase in the heart, liver, and spleen of chickens infected with strain SDJN0105 [21] and in the heart, liver, spleen, thymus, and BF of chickens infected with SDDM-4/15 [27, 46, 55]. We found that the expression level of TNF-α was significantly increased in the heart, lung, cecal tonsils, duodenum, proventriculus, and thymus of chickens infected with the pathogenic strain CH/HNJZ/2015 and only in the cecal tonsils of chickens infected with the non-pathogenic strain ON1 at 48 hpi compared to the control group. The expression of TNF-α was significantly different between the CH/HNJZ/2015 group and the ON1 group, indicating its vital role in the pathogenicity of FAdV-4.

Expression of cytokines of adaptive immunity

IL-2 and IL-4

Meng et al. observed that the expression of IL-2 peaked at 2 dpi in the heart (8.47-fold), liver (6.33-fold), and spleen (6.53-fold) and that the expression of IL-4 peaked at 1 dpi in the liver (5.63-fold) and in the spleen (8.26-fold) of 28-old-day chickens infected with the pathogenic strain SDJN0105 through the IM route [21]. Wu et al. inoculated 21-day-old SPF chickens with the FAdV-4 strain SX17 via the IM route and found that the expression level of IL-2 increased significantly starting from 3 dpi and peaked at 4 dpi in the liver (57.75-fold) and thymus (31.79-fold) of the infected chickens. The expression level of IL-2 in both organs dropped significantly at 5 dpi. The expression of IL-4 peaked at 3 dpi in the liver and thymus (6.92-fold and 8.19-fold) [56].

Our research showed that the mRNA expression level of IL-2 increased significantly in the heart, lungs, and pancreas at 24 hpi and in the heart, lungs, and BF at 48 hpi in chickens infected with the pathogenic strain CH/HNJZ/2015. However, in ON1-infected chickens, the expression of IL-2 was significantly upregulated only in the lungs at 24 and 48 hpi. The expression level of IL-4 in chickens infected with CH/HNJZ/2015 increased significantly in the BF at 24 hpi and in the heart, spleen, lungs, BF, duodenum, and thymus at 48 hpi (Table 3). In chickens infected with ON1, IL-4 was only significantly upregulated in the BF at 24 and 48 hpi and in the duodenum at 48 hpi.

IL-2 is a T cell growth factor that is approved in treatments of human metastatic melanoma and renal cell carcinoma. It exhibits the ability to increase the population of peripheral blood CD4$^+$ and CD8$^+$ T cells [57]. The

Table 3 Changes in the expression of cytokines of the adaptive immune system in different organs of chickens infected with FAdV-4

Cytokine	Strain	Route	Organ						Ref
			Heart	Liver	Spleen	Lung	Bursa of Fabricius	Thymus	
IL-2	SDJN0105	IM	↑ (2d)	↑ (2d)	↑ (2d)				[21]
	SX17			↑ (3&4d)				↑ (3&4d)	[56]
	CH/HNJZ/2015		↑ (24&48h)	--	--	↑ (24&48h)	↑ (48h)	--	U/D
	ON1		--	--	--	↑ (24&48h)	--	--	
IL-4	SDJN0105	IM		↑ (1d)	↑ (1d)				[21]
	SX17			↑ (3d)				↑ (3d)	[68]
	CH/HNJZ/2015		↑ (48h)	--	↑ (48h)	↑ (48h)	↑ (24&48h)	↑ (48h)	U/D
	ON1		--	--	--	--	↑ (24&48h)	--	
IFN-γ	SDJN0105	IM			↑ (2d)				[21]
	SD0828	IM			↑ (1d)				[22]
		Oral			--				
	HN/151025	IO			↓ (36h)		↓ (36h) ↑ (7d)		[20]
	ON1	IM		↑ (3d)	↓ (10d)				[29]
	CH/HNJZ/2015		↑ (48h)	↑ (48h)	--	↑ (24&48h)	--	--	U/D
	ON1		↑ (48h)	--	--	--	--	--	

IM, intramuscular; IO, intraocular; ↑, significant upregulation; ↓, significant downregulation; h, hours postinfection; d, days postinfection; --, no significant change; Ref, reference; U/D, unpublished data

decreased expression of IL-2 in the later stage of FAdV-4 infection might be caused by the severe damage in the immune organs. A previous study by Schonewille et al. indicated a pronounced decrease in single positive CD3^{+}, CD4^{+}, and CD8^{+} cells in the spleen and CD4^{+} CD8^{+} cells in the thymus of chickens challenged intramuscularly with the pathogenic FAdV-4 strain AG234 [6]. It was also demonstrated that FAdV-4 infection inhibited antibody production induced by inactivated vaccines against Newcastle disease virus and avian influenza virus [46]. It has been speculated that the significant downregulation of IL-2 might be connected to the immunosuppressive activities of FAdV-4. Previous research indicated that IL-2 has a positive effect on clearance of Newcastle disease virus [58], and coexpression of IL-2 in the IBDV DNA vaccine increases T lymphocyte proliferation and IFN-γ production [59]. IL-4 is a TH2 cytokine that regulates M2 macrophages in chickens [60]. M2 macrophages are involved in anti-inflammatory type 2 responses, which might negatively regulate the type 1 proinflammatory responses directed by IFN-γ. M2 macrophages also play important roles in tissue repair and metabolic functions [61]. A previous study showed that co-administration of IL-4 with an NDV DNA vaccine induced a stronger antibody response and enhanced humoral immunity [62].

IFN-γ

IFN-γ is the only type II interferon known to be responsible for activation of macrophages and neutralization of viral replication [56, 59]. It also enhances the activation of both MHC-I and MHC-II molecules and activates Th1-type immune responses. The adjuvant property of IFN-γ has been shown to increase the efficacy of vaccination against AIV, NDV, and Marek's disease virus (MDV) [56, 60, 61].

The expression of IFN-γ has been investigated in both pathogenic and non-pathogenic FAdV-4 infection (Table 3). A 188.07-fold increase in IFN-γ was observed in the spleen of chickens infected with the pathogenic FAdV-4 strain SDJN0105 at 2 dpi [21]. The expression of IFN-γ was significantly upregulated in the spleen (104.17-fold) of chickens infected with the pathogenic FAdV-4 strain SD0828 at 1 dpi via the IM route, but no significant difference was observed in chickens infected via the oral route. Infection with SD0828 via the oral route did not cause a significant change in the expression of IFN-γ [22]. The expression levels of IFN-γ in the spleen and BF of chickens infected intraocularly with the pathogenic strain HN/151025 were significantly lower than in the control group at 36 hpi. However, in the BF, it was significantly higher than in the control group at 7 dpi [20]. The mRNA expression level of IFN-γ was studied in chickens infected with the non-pathogenic strain ON1 via the IM route as well. The expression increased significantly in the liver at 3 dpi, dropped significantly in the spleen at 10 dpi, and remained at baseline in the cecal tonsil throughout the entire experiment [29].

Our research indicated that the expression level of IFN-γ increased significantly in the lungs of chickens infected with CH/HNJZ/2015 at 24 hpi and in the heart, liver, lungs, cecal tonsils, duodenum, and proventriculus at 48 hpi. In chickens infected with ON1, the expression level of IFN-γ increased

in the cecal tonsils at 24 hpi and in the heart, duodenum, and proventriculus at 48 hpi.

The expression of antiviral cytokines

Interferon responses (IFN-α and IFN-β)

Both IFN-α and IFN-β are type I interferons. The innate immune system blocks viral replication mainly by producing type I interferons (especially IFN-α) in the early stages of viral infection. The antiviral properties of type I interferons are essentially mediated by the induction of hundreds of IFN-stimulated genes (ISGs) in the neighboring cells. Pre-treating chickens with IFN-α before AIV challenge significantly reduced virus replication in both chicken and turkey-origin lung cells [63].

The expression of IFNs was found to be upregulated significantly in different studies, indicating their importance in the immune responses induced by FAdV-4 infection (Table 4). A 104-fold increase in IFN-α and a 602.6-fold increase in IFN-β were observed in the spleen of chickens infected with the pathogenic FAdV-4 strain SDJN0105 at 2 dpi [21]. IFN-α and IFN-β expression was significantly upregulated (171.01- and 177.69-fold, respectively) in the spleen of chickens infected intramuscularly with the pathogenic FAdV-4 strain SD0828 at 1 dpi, but no significant difference was observed in the expression of the two cytokines in chickens infected via the oral route [22]. Chickens infected with the pathogenic FAdV-4 strain SX17 via the IM route showed significant increases in IFN-α and IFN-β expression in the liver and the thymus at 4 dpi [56].

In our research, IFN-α levels increased significantly in the heart, liver, lungs, and BF of chickens infected with strain ON1 via the IM route at 24 hpi, whereas no statistically significant difference was observed in chickens infected with CH/HNJZ/2015 at 24 hpi. Subsequently, the expression level of IFN-α in chickens infected with CH/HNJZ/2015 increased significantly at 48 hpi in the heart, lungs, BF, and duodenum compared to the control group, whereas in the ON1 group, it returned to background levels. IFN-β was significantly upregulated in the lungs, thymus, and pancreas of chickens infected with CH/HNJZ/2015 at 24 hpi and in the liver, spleen, lungs, cecal tonsils, BF, duodenum, proventriculus, and pancreas at 48 hpi. On the other hand, IFN-β increased significantly only in the spleen of chickens infected with ON1 at 24 hpi.

Summing up the observations from different studies, the IFN-β response shows the most variation between pathogenic and non-pathogenic FAdV-4 infections. Therefore, studies of the function of IFN-β could contribute to our understanding of the different immune responses induced by infections with pathogenic and non-pathogenic FAdV-4 strains.

Bringing it all together: the role of cytokines in FAdV-4 infection

Studying the interaction of the host immune system with the virus is pivotal for understanding the pathogenicity of FAdV-4 and developing strategies to control FAdV-4 infection. Despite sporadic reports on cytokine expression after FAdV-4 infection, there has not yet been a systematic study on the immune response induced by FAdV-4 infection,

Table 4 Changes in the expression of antiviral cytokines in different organs of chickens infected with FAdV-4

Cytokine	FAdV-4 strain	Route	Organ							Ref
			Heart	Liver	Spleen	Lung	Cecal tonsil	Bursa of Fabricius	Thymus	
IFN-α	SDJN0105	IM			↑ (2d)					[21]
	SD0828	IM			↑ (1d)					[22]
		Oral			--					
	SX17	IM		↑ (4d)					↑ (4d)	[56]
	CH/HNJZ/2015		↑ (48h)	--	--	↑ (48h)	--	↑ (48h)	--	U/D
	ON1		↑ (24h)	↑ (24h)	--	↑ (24h)	--	↑ (24h)	--	
IFN-β	SDJN0105	IM			↑ (2d)					[21]
	SD0828	IM			↑ (1d)					[22]
		Oral			--					
	SX17	IM		↑ (4d)					↑ (4d)	[56]
	CH/HNJZ/2015		--	↑ (48h)	↑ (48h)	↑ (24&48h)	↑ (48h)	↑ (48h)	↑ (24h)	U/D
	ON1		--	--	↑ (24h)	--	--	--	--	

IM, intramuscular; IO, intraocular; ↑, significant upregulation; ↓, significant downregulation; h, hours postinfection; d, days postinfection; --, no significant change,; Ref, reference; U/D, unpublished data

especially on the expression of different cytokines induced by FAdV-4 strains with different pathogenicity. Cytokines are a group of soluble proteins that play diverse and sometimes redundant roles in the immune system. Inflammatory cytokines are classified as ILs, IFNs, TNGs, TGFs, colony-stimulating factors (CSFs), and chemokines. The best-studied cytokines in the case of FAdV-4 infection are ILs, IFNs, and TNFs. The exact function of a cytokine is determined by when and where it is produced and where its receptors are expressed. Cytokines often influence their own production as well as the production of other cytokines to form a complex cytokine network. Most cytokines are functionally pleiotropic and sometimes can have a negative impact on the host. Connections are often found between overexpression of certain cytokines, such as IL-1β and TNF-α, and inflammatory damage. Sometimes invading pathogens even hijack cytokines such as IL-6 to breach the line of immune defense. However, it is difficult to conclude whether a cytokine contributes to or protects hosts from the tissue damage caused by viral attack. The net effect of cytokines largely depends on the place, timing, and duration of their action.

After the initial recognition of FAdV-4 by TLRs, NLRP3, and MDA5, intracellular cascades of cytokine expression are triggered by nuclear translocation of transcription factors such as NF-κB. Notably, cytokines such as IL-1β and TNFs in turn activate NLRP3 inflammasomes to increase production of proinflammatory cytokines [64]. There is accumulating evidence that pathogenic FAdV-4 infection induces significant upregulation of both pro- and anti-inflammatory cytokines in the major organs of chickens. The rapid accumulation of proinflammatory cytokines released by immune effector cells has been suggested to cause organ failure and even death [65, 66]. Severe inflammatory damage such as pericardial effusion is also observed in FAdV-4-infected chickens, and it can therefore be assumed that an excessive inflammatory response induced by FAdV-4 infection could result in organ exhaustion and, ultimately, death. For example, extremely congested and swollen spleens are very often observed in chickens infected with pathogenic FAdV-4. At the same time, it has been found that almost all of the inflammatory cytokines mentioned above are significantly upregulated in the spleen of chicken infected with pathogenic FAdV-4.

For comparison, cytokine expression in chickens infected with the non-pathogenic FAdV-4 strain ON1 was assessed by Grgić and coworkers as well as by our research group. Despite the 98.44% sequence identity of the non-pathogenic strain ON1 and pathogenic strain CH/HNJZ/2015, the levels of mRNA transcripts encoding cytokines differ greatly between chickens infected with ON1 and those infected with pathogenic strains. For example, the proinflammatory cytokines IL-8 and IL-18 were significantly upregulated in various organs in chickens infected with pathogenic strains

but remained at baseline for most organs in chickens infected with ON1. Furthermore, the expression level of the antiviral cytokine IFN-β also increased significantly in chickens infected with the pathogenic FAdV-4 strain compared to the non-pathogenic strain. The analysis of differences in the immune response to infection might facilitate further studies comparing the pathogenicity of the two strains.

How cytokines act in the immune response is important to determine their role as a friend or a foe. Immunoregulatory cytokines have long been of therapeutic interest in the prevention and treatment of many diseases. Cytokines used as vaccine adjuvants enhance the immune response by upregulating the humoral and cellular responses to the antigen [67]. Co-administration of viral antigens and vectors encoding cytokines leads to increased antibody titers in both mammals and birds. IL-1β, 2, 4, 6, and 18 have demonstrated potential as vaccine adjuvants or treatment aids for Newcastle disease, AIV, and IBDV. However, their possible application in FAdV-4 infection has not been investigated.

Conclusions

The immune response often acts as a double-edged sword. It reacts to viral infection to eliminate the invading pathogen, in part by activation of inflammatory cytokines. However, excessive inflammation often leads to negative outcomes. Identification of specific genes of FAdV-4 that are associated with up- or downregulation of certain cytokines will undoubtedly contribute to our understanding of the pathogenesis of FAdV-4. A reverse genetic platform for FAdV-4 could provide a tool to make FAdV-4 mutants carrying specific genes of pathogenic or non-pathogenic FAdV-4 strains. Comparing the cytokine expression induced by different FAdV-4 mutants will facilitate the elucidation of FAdV-4 pathogenesis.

Declarations

Conflict of interest The authors declare no conflict of interest.

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