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Full Length Article

In-vitro assessment of differential cytokine gene expression in response to infections with Egyptian classic and variant strains of highly pathogenic H5N1 avian influenza virus

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Abstract In Egypt, two distinct genetic groups of HPAI H5N1 viruses are co-circulating: classic 2.2.1/C sub-clade and antigenic drift variant 2.2.1.1 clade isolated from vaccinated poultry flocks. The response of chicken innate immunity to both genotypes is not investigated, so far. In this study, expression of immune related genes (IL1b, IL4, IL6, IL8, IL10, IL18, IFNα and IFNγ) after infecting chicken macrophage cell line (HD11) and chicken peripheral blood Mononuclear cells (PBMC) with a classic and a variant strains was assayed using quantitative reverse-transcription real-time polymerase chain reaction assays (qRT-PCR). In HD11, the variant strain induced higher levels of IL1b and IL8 at 6 hours post infection (hpi), IL4 at 24 / 48 hpi and IFNα at 48 hpi than the classic strain. Conversely, the classic strain induced about 10-fold increase of IFNγ at 24 and 48 hpi and the virus replicated at higher level than the variant strain. The results of PBMC infection were similar to that reported from HD11 except for IFNγ gene expression that was higher at variant strain infected cells than that infected with the classic strain. After 24hpi skewing the innate immune response toward anti-inflammatory (humoral-associated) cytokines was different between HD11 (through IL4) and PBMC (through IL10). To sum up, the classic strain produced less cytokines

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which may indicate adaptation to evade the recognition by the innate immune system and explain its higher pathogenicity.

1. Introduction

Highly pathogenic avian influenza virus subtype H5N1 (HPAIV H5N1) is a worldwide devastating disease of poultry, which presents a potential pandemic threat [1]. Since its emergence in 1997 in Hong Kong, the virus spread to more than 60 countries and finally became endemic in poultry populations at Bangladesh, China, Egypt, Indonesia and Viet Nam [2]. In case of Egypt, losses in poultry industry since 2006 was estimated to exceed $1 billion due to culling or death of over 30 million birds [3]. According to the WHO, the virus was spilled over to 173 persons and caused deaths in 63 patients by the 26th of November, 2013 [4]. Vaccination of commercial poultry against the HPAIV H5N1 using different H5 vaccines was a milestone in the control of the disease in Egypt. The reduced number of outbreaks after the first wave in 2006 in poultry was attributed to the effectiveness of these vaccines to interrupt the circulation of the virus particularly in the commercial sector [3]. Since 2007, a dramatic increase in the number of infected flocks despite vaccination was reported [5].

Phylogenetic analyses of the Egyptian H5N1 viruses indicated co-circulation of two distinct genetic groups. The first group belongs to the 2.2.1/C subclade, also known as classic group, is very close to the predecessor 2.2.1 viruses introduced into Egypt in early 2006. These viruses were isolated from non-vaccinated backyard birds as well as from human [6]. They were also able to induce clinical disease and mortality in improperly vaccinated chickens in small-scale commercial farms [7]. The second genetic group classified separately in a unique 2.2.1.1 clade. Viruses in this clade represent the antigenic drift variants isolated from vaccinated birds and harbour major changes in immunogenic epitopes of the hemagglutinin (HA) protein [6]. Experimental challenge studies showed that the classic group caused mild clinical signs in chickens vaccinated with homologous or heterologous H5 vaccines; however it was excreted for long periods of time. On the contrary, the variant strains caused up to 100% mortality in chickens vaccinated with heterologous vaccines and virus excretion was limited in birds vaccinated with homologous H5N1 vaccines [8]. Accordingly, since 2011 no variant virus was isolated and the classic strains are the predominant genotype in Egypt due to probably adoption of more genetically related homologous vaccines in the commercial poultry [6].

Much emphasis has been placed on the humoral immunity but the response of innate immune system of chickens to infections with H5N1 strains has not been adequately studied [9]. The current dogma of the immunology states that the innate immune response is the first line of defence of a host against microbial invasion [10]. An essential component of the innate immune system is cytokines which are triggered upon stimulation of host-cells with a micro-organism. They orchestrate innate and adaptive antiviral defence mechanisms to eliminate (e.g. influenza virus) infections from the host [11]. According to their function, three classes of cytokines are mostly important (1) proinflammatory cytokines such as interleukin-1β (IL-1β), Interleukin-6 (IL6), IL8 and tumour necrosis factor-α (TNF-α) that play a role in the induction of inflammation during the course of infection, (2) T-Helper 1 (Th1) associated cytokines including IL18 and IFNγ that regulate and induce cell mediated immune (CMI) response and (3) anti-inflammatory/Th2 cytokines like IL4 and IL10 that involved in the induction and regulation of humoral immune response [9]. In comparison to mammals, repertoire of cytokines in chicken was not fully understood until recently. A considerable number of chicken immune-related molecule orthologs have been identified and quantification of cytokine messenger RNA (mRNA) expression levels using quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) improved our knowledge on the host-virus interaction [12]. In human or mammal models, extensive literatures have been published on the molecular viral mechanisms involved in the H5N1 pathogenesis [13] but very little is known about the host-influenza-interaction in chickens particularly the regulation of the innate immune response by HPAIV H5N1 immune-escape variants.

In the present study, HD11cell line and chicken PBMCs were used to study the regulation of cytokines upon infections with a classic and a variant HPAI strains isolated from chickens in Egypt.

2. Materials and methods

2.1. H5N1 strains propagation

Two viruses were obtained from the influenza virus repository of the Reference Laboratory for Quality Control on Poultry Production (RLQP), Egypt. A/chicken/Egypt/0963S-NLQ P/2009(H5N1), GenBank accession number HQ198269 belongs to the variant 2.2.1.1 clade and A/chicken/ Egypt/ 121/2012(H5N1), GenBank accession number JQ858483 belongs to the classic 2.2.1/C subclade. Both viruses were propagated in 9 day-old specific pathogen free embryonated chicken eggs according to the standard protocol [14]. All procedures were performed in BSL3 laboratory facilities at the National Institute of Animal Health (NIAH), Japan. Viral titters were expressed as mean tissue culture infectious dose ($TCID_{50}$) using HD11 cell line for each strain according to Reed and Muench [15].

2.2. HD11 cell line propagation and infection

HD11 cells were kindly provided by Dr. John Adams (the Cedars-Sinai Medical Centre, Los Angeles, CA, USA). Cell were counted and diluted in 2 mL growth media per well of 6-well plates to get 1.5×10^6 cell per well and incubated for 24 hrs to form a confluent sheet. After 24 hrs the media were removed and cells were washed with PBS. Cells were collected from one well of 6 well plate with trypsin and were counted to

calculate the dose of the infection. A total volume of 0.5mL infection media contain the filtered viral allantoic fluid (0.05TCI50/cell) were added to every well and incubated for 1 hour with gently shaking every 15 min then the media was removed and cells were washed with PBS followed by addition of 2 mL of MEM medium (Gibco; Carlsbad, CA, USA). Plates were incubated at 37 °C in 5% CO_2 incubator. HD11 cultures were used in parallel without the addition of viral allantoic fluid (VAF) as negative control. Samples (cells and supernatant) were collected at 6, 24 and 48 hr post infection (hpi) for RNA extraction as described below.

2.3. PBMC isolation, culturing and infection

Heparinized blood was collected form 10 week-old chickens and mixed with equal volume of PBS, then layered drop by drop over equal volume of Ficoll-Paque (GE health care, Sweden) followed by centrifugation at 400×*g* for 30 min without brake. PBMC were removed at the mononuclear cell interface carefully and washed twice with PBS and then subjected to centrifugation at 100×*g* for 10 min. The resultant pellet was re-suspended in complete RPMI media (Gibco, Carlsbad, CA, USA) containing 10% FBS. PBMC were grown overnight at cell density 10^7 per well of 12-well tissue culture plates and incubated at 37 °C in 5% CO_2 incubator. After overnight growth, non-adherent cells were removed by washing the monolayers with PBS to enrich the cultures for adherent macrophages, monocytes and dendritic cells. One PBMC culture was trypsinized and the cells were counted to calculate the required amount of VAF to get infection dose of 0.05TCID50/cell. Growth media was replaced with infection media containing complete RPMI but without serum and with the addition of filtered VAF. Negative control PBMC cultures without the addition of VAF were incubated in parallel. Culture plates were gently shaken every 15 min for 1 hr then the media was replaced with RPMI supplemented with 0.2% FBS. Cultures were incubated at 5% CO_2 and RNA was extracted from the cell monolayer and supernatant at 6, 24, and 48hpi.

2.4. Total RNA extraction

Total RNA was isolated from infected and control HD11 and PBMC cells and supernatant at each time point using RNeasy mini RNA Purification kit and DNase treatment with QIAGEN®™ RNA purification kits to purify RNA from DNA contamination following manufacturer's instructions (Qiagen Inc., Valencia, CA, USA). RNA in each sample was quantified Using NanoDrop-1000 (Thermoscientific, Wilmington, DE).

2.5. qRT-PCR

qRT-PCR was performed using Quantitect probe RT-PCR (Qiagen, Inc. Valencia, CA, USA) according to the manufacturer recommendations. Primers and probes were selected for amplification of IL1b, IL6 and IFNγ [16], IL4 and IL10 [17], IL8 and IL18 [18], and IFNα [19] as shown in Table 1. qRT-PCR runs were performed using 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). PCR conditions were the same for each targeted gene of different cytokines as follows: 30 min at 50 °C, 95 °C for 15min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 1min. For detection of AIV H5 [20], the thermoprofile was 30 min at 50 °C, 95 °C for 15 min, followed by 40 cycles of 95 °C for 10 s, 54 °C for 30 s and 72 °C for 30 sec.

Absolute quantification for viral RNA was calculated using tenfold-serially diluted RNA from known $TCID_{50}$ classic and variant strains. The standard curves were created automatically by plotting the threshold level of fluorescence (Ct values) against the $TCID_{50}$ for every dilution. The Ct values of the unknown samples were then compared to the Ct values of the standards. The starting template quantities for the unknown samples could be estimated in the present study using standard curve generated with every PCR run. For relative quantification of cytokines mRNA, amplification data of cytokines were normalized against 28s RNA [16] and fold change of cytokines mRNA gene expression of infected cells compared to non-treated cells was calculated as previously published [21].

3. Results

3.1. Virus titration

Serial titrations of both viral strains were performed in HD11 cell line and $TCID_{50}$ titres were determined. The titer of the variant strain was calculated as $4\times10^{6.4}$ TCID50/mL while the classic strain was $4\times10^{5.6}$ TCID50/mL. The variant strain grew at lower levels than the classic strain in HD11 at all-time points reaching $10^{5.5}$ and $10^{6.8}$ $TCID_{50}$/mL at 48 hpi for the variant and classic strains, respectively (data not shown). While in PBMCs, both viruses grew at similar $TCID_{50}$ levels at different time point (data not shown).

3.2. Cytokines mRNA gene expression using HD11 cell line (Table 2)

Cytokines mRNA gene response was detected at 6, 24 and 48 hpi after infection using qRT-PCR (Fig. 1). The pro-inflammatory cytokines IL1b and IL8 were expressed at high levels and early at 6 hpi, while expression of IL4 and IFNγ increased later at 24 and 48 hpi compared with the non-infected cells. At 6 hpi, the variant strain increased the IL8, IL1b, IL4 and IL10 expression levels by 6.5, 4.2, 1.1 and 1.0-fold, respectively compared to those levels induced by the classic strain. Likewise, higher folds of about 1.0 and 7.1 at 24 hpi and 3.9 and 5.7 at 48 hpi for IL1b and IL4, respectively were obtained by the variant strains. Conversely, at 24 and 48 hpi about 18.0 and 14.0 higher folds of IFNγ respectively were induced by the classic strains than those obtained by the variant strain. IFNα gene expression levels were increased only at 48 hpi by the variant strain infected cells while other cytokines showed no marked changes (Fig. 1).

3.3. Cytokines mRNA gene expression using PBMC cell culture (Table 3)

The level of cytokines mRNA gene expression markedly upregulated by both strains at each time point particularly

Table 1 Sequences of the primers and probes used in quantitative qRT-PCR.

Gene	Name	Sequence (5′–3′)	Refs.
IL1B	F-Primer	GCTCTACATGTCGTGTGTGATGAG	Kaiser et al. [16]
	R-Primer	TGTCGATGTCCCGCATGA	
	Probe	F AM-CCACACTGCAGCTGGAGGAAGCC-(TAMRA)	
IL4	F-Primer	AACATGCGTCAGCTCCTGAAT	Kumar et al. [17]
	R-Primer	TCTGCTAGGAACTTCTCCATTGAA	
	Probe	FAM-AGCAGCACCTCCCTCAAGGCACC-TAMRA	
IL6	F-Primer	GCTCGCCGGCTTCGA	Kaiser et al. [16]
	R-Primer	GGTAGGTCTGAAAGGCGAACAG	
	Probe	FAM-AGGAGAAATGCCTGACGAAGCTCTCCA-TAMRA	
IL8	F-Primer	GCCCTCCTCCTGGTTTCA G	Kogut et al. [18]
	R-Primer	TGGCACCGCAGCTCATT	
	Probe	FAM-TCTTTACCAGCGTCCTACCTTGCGACA-TAMRA	
IL10	F-Primer	CATGCTGCTGGGCCTGAA	Kumar et al. [17]
	R-Primer	CGTCTCCTTGATCTGCTTGATG	
	Probe	FAM-CGACGATGCGGCGCTGTCA-TAMRA	
IL18	F-Primer	AGGTGAAATCTGGCAGTGGAAT	Kogut et al. [18]
	R-Primer	ACCTGGACGCTGAATGCAA	
	Probe	FAM-CCGCGCCTTCAGCAGGGATG-TAMRA	
IFNα	F-Primer	GACAGCCAACGCCAAAGC	Eldaghayes et al. [19]
	R-Primer	GTCGCTGCTGTCCAAGCATT	
	Probe	FAM-CTCAACCGGATCCACCGCTACACC-TAMRA	
IFNγ	F-Primer	GTGAAGAAGGTGAAAGATATCATGGA	Kaiser et al. [16]
	R-Primer	GCTTTGCGCTGGATTCTCA	
	Probe	FAM-TGGCCAAGCTCCCGATGAACGA-TAMRA	
28S	F-Primer	GGCGAAGCCAGAGGAAACT	Kaiser et al. [16]
	R-Primer	GACGACCGATTTGCACGTC	
	Probe	FAM-AGGACCGCTACGGACCTCCACCA-TAMRA	
AIV (H5)	LH1-Primer	ACATATGACTACCCACARTATTCAG	Slomka et al. [20]
	RH1-Primer	AGACCAGCTAYCATGATTGC	
	Probe	FAM-TCWACAGTGGCGAGTTCCCTAGCA-TAMRA	

Table 2 Cytokines mRNA fold change at 6, 24 and 48 h after infection of HD11 cell line with 0.05 TCID50 per cell.

Cytokines	6 h		24 h		48 h	
	Classical	Variant	Classical	Variant	Classical	Variant
IL1b	23.96405	28.12147	1.261552	2.211308	1.198143	5.088005
IL4	1.3297	2.39197	13.8864	21.0303	20.8171	26.4904
IL6	1.11891	1.2886	1.30279	0.99481	1.20497	1.88296
IL8	4.63992	11.1255	0.48609	0.55826	0.51939	0.82599
IL10	1.32648	2.25793	0.29968	0.26521	0.07471	0.14839
IL18	2.90291	2.46844	0.42647	0.14876	0.35792	0.18233
IFNγ	2.61389	3.52079	26.5309	8.56112	22.4773	8.5185
IFNα	1.07482	1.00292	0.95754	0.94606	1.91906	3.24204

Table 3 Cytokines mRNA fold change at 6, 24 and 48 h after infection of PBMC with 0.05 TCID50 per cell.

	6 h after infection		24 h after infection		48 h after infection	
	Classical	Variant	Classical	Variant	Classical	Variant
IL1b	63.3819	111.4691	9.1883	20.8157	4.0424	21.6111
IL4	2.8036	1.2879	0.9764	3.1199	1.1037	3.0111
IL6	184.3879	506.8096	13.5226	24.804	12.4416	12.4554
IL8	219.4274	136.5232	34.0386	30.6009	10.1944	35.8152
IL10	4.5732	84.4368	4.1592	70.3608	3.5373	26.3768
IL18	2.6036	5.1437	1.0786	1.0449	0.3396	0.4444
IFNγ	4.5732	84.4368	0.6596	5.6859	2.0669	6.7473
IFNα	0.4411	1.1423	0.4180	1.7633	0.2595	0.8520

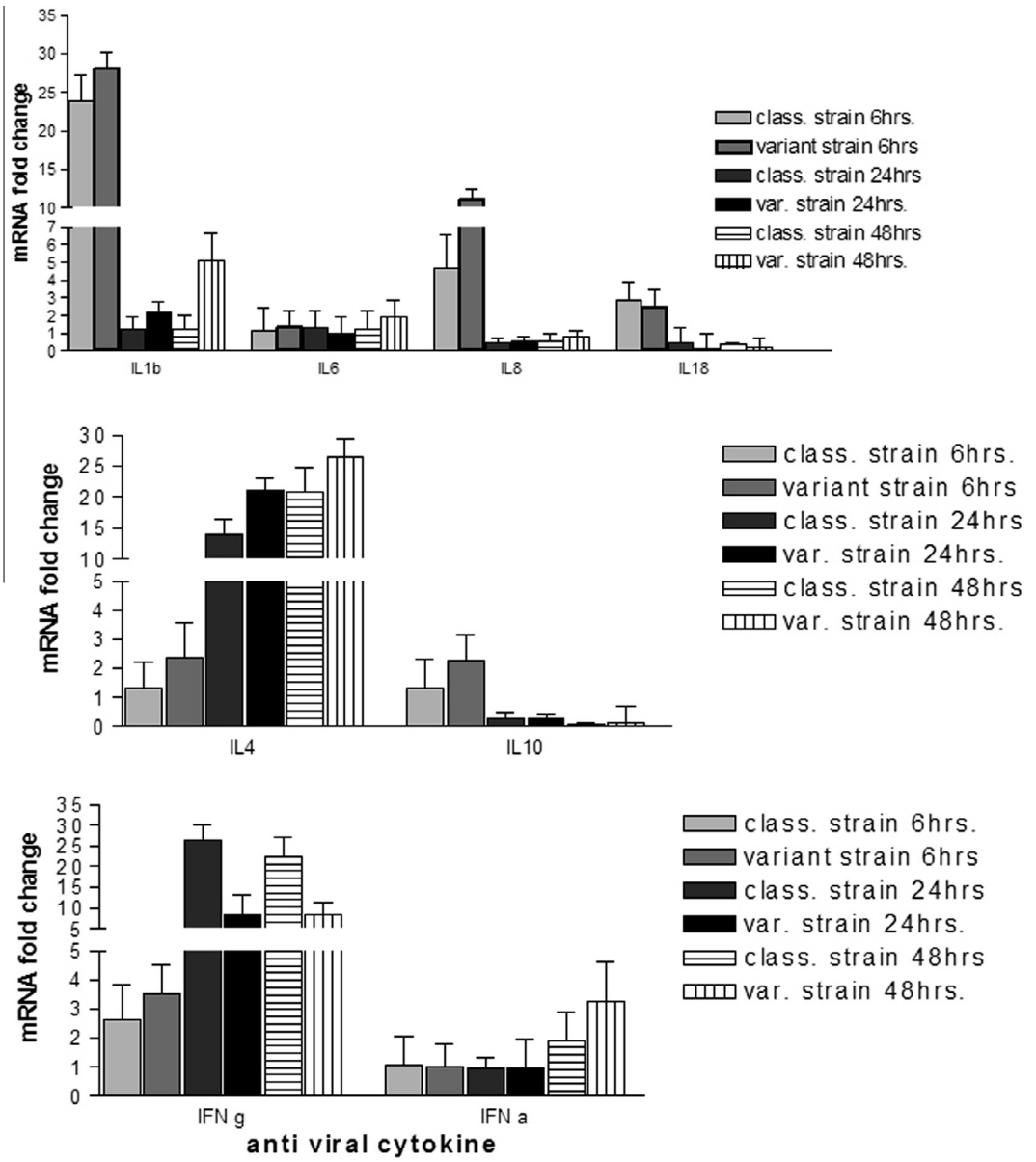

Fig. 1 Cytokines mRNA fold change at 6, 24 and 48 h post infection (hpi) of HD11 cell line with Egyptian H5N1 variant (A/chicken/Egypt/0963S-NLQP/2009) and classical (A/chicken/Egypt/121/2012) strains. mRNAs of IL1b, IL6, IL8, IL4, IL10, IFNγ and IFNα were measured by qRT-PCR in HD11 infected cells. Data show fold change compared with uninfected control HD11 cells. Data represent the mean; error bars show SE.

IL1b, IL6 and IL8 at 6 hpi (Fig. 2). The variant strain at 6hpi elicited 48.1 to 322.4 folds higher of the IL1b, IL6, IL10 and IFNγ than the classic strain. Conversely, at 6 hpi the classic strain elicited 1.5 and 82.9 folds of IL4 and IL8, respectively higher than the variant strain. At 24hpi and 48hpi, higher levels of expression of most of cytokines induced by the variant strain than those induced by the classic strain were observed (Fig. 2). IL8 showed higher mRNA gene expression after infection of PBMC with the classical strain than that infected with variant strain at 6 hpi but at 48 hpi the variant strain infected PBMC showed higher IL8 mRNA gene expression (Fig. 2). PBMC infected with the variant strain showed marked higher IFNγ mRNA gene expression than that infected with the classic strain. The classic strain did not regulate IFNα mRNA gene expression similar to the negative control cells but infected PBMC with the variant strain was slightly upregulated (Fig. 2).

4. Discussion

Virulence of HPAIV H5N1 can be modulated by the virus-host immune system interaction [22,23]. Innate immune responses via interferon and other cytokines can limit or eliminate influenza virus infections. Variations of cytokines expression' pattern between influenza virus serotypes (e.g. H5N1 and H1N1) [24] or even within the same subtype (e.g. H5N1 of wild bird vs. of chickens origins) were intensively elucidated in several literatures [12]. Macrophages are an important cellular

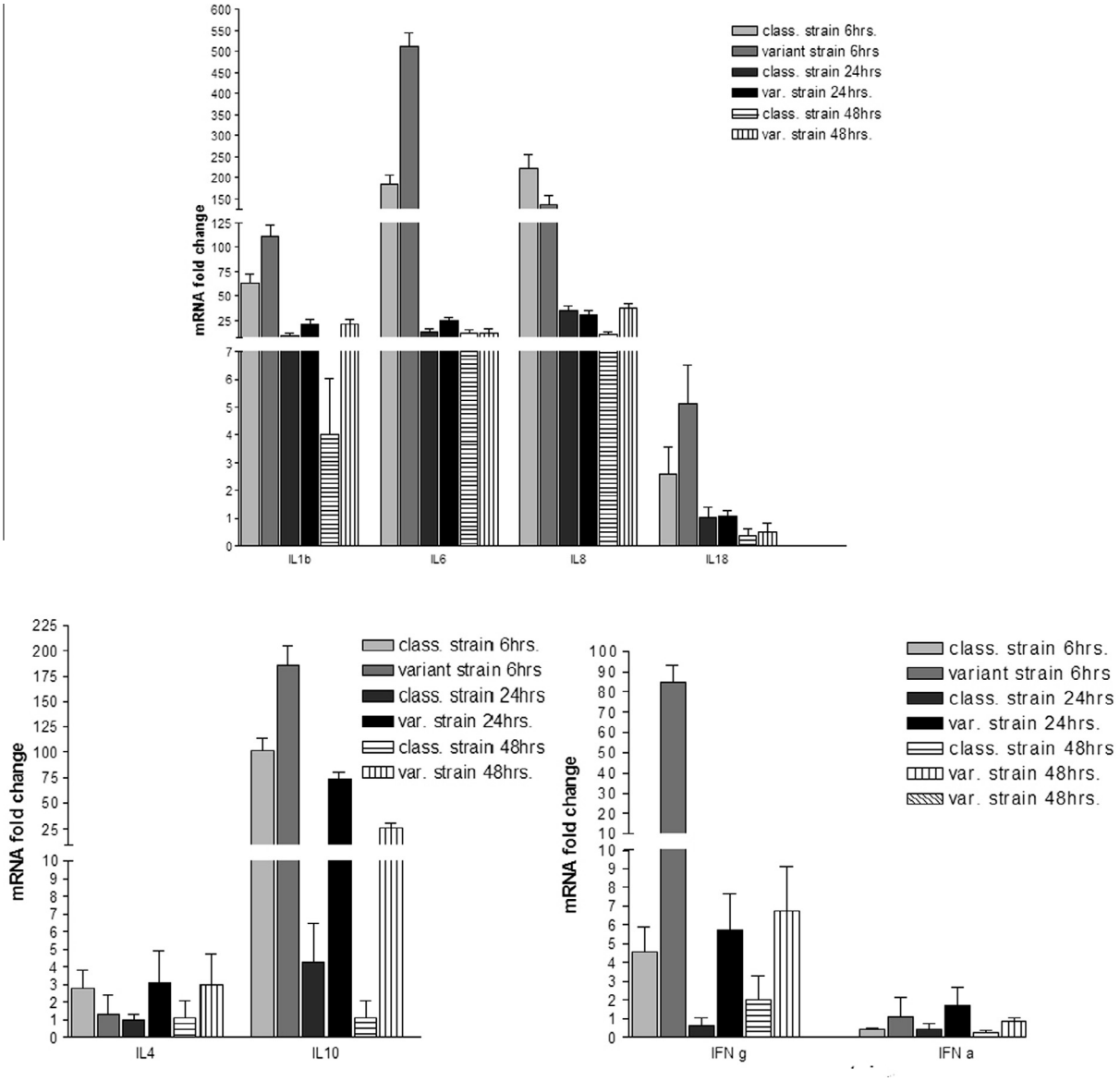

Fig. 2 Cytokines mRNA fold change at 6, 24 and 48 h post infection (hpi) of PBMC cell line with Egyptian H5N1 variant (A/chicken/Egypt/0963S-NLQP/2009) and classical (A/chicken/Egypt/121/2012) strains. mRNAs of IL1b, IL6, IL8, IL4, IL10, IFNγ and IFNα were measured by qRT-PCR in PBMC infected cells. Data show fold change compared with uninfected control PBMC cells. Data represent the mean; error bars show SE.

component of the immune system and play a primary role in the development of both innate and adaptive immune responses. The use of chicken macrophage cells is an optimal system to study the interaction between HPAIV H5N1 and the immune system of birds, nevertheless only limited numbers of studies have been published [25].

In this study, in HD11 cell line both virus genotypes up-regulated the expression level of cytokines, particularly at 6 hpi, compared to the negative control cells. At early stage of infection (6hpi) expression levels of the pro-inflammatory cytokines IL1b and IL8 were remarkably increased, but at later stages of infection (24 and 48hpi) the IL4 and IFNγ levels were the highest, while IFNα expression showed no marked change at early stage. Such results coincide with that of Watanabe et al. [26] who recorded up-regulation of IL1b, IL8, IFNγ and IL18 in HD11 cell line infected with an HPAIV H5N1 [27]. Also, low expression of IFNα was correlated with high viral titre and prolonged shedding time in chickens [28]. Different expression patterns between both viruses were observed. Generally, the variant strain elicited higher IL1b, IL4, IL8 and IFNα but the classic strain induced higher IFNγ. This may explain the low replication titter of the variant strain in HD11and the positive correlation between viral titre and IFNγ expression recorded *in vitro* [29]. These data support the field observation of the higher virulence of classical strain comparing to variant strain. As the variant strain induced higher innate immune response that represented by higher IL1b,IL8 and IFNα and the immunity skewed toward humeral immune response represented by IL4 that can efficiently reduce the pathogenicity of the virus and its titre, while classical strain induced lower innate immunity with higher IFNγ that correlated with higher viral titre. These finding agree with that of

Friesenhagen et al. [32], who suggested that much stronger inflammatory response of human macrophages to infection with low pathogenic virus than highly pathogenic avian influenza.

Avian species have a similar but not identical network of macrophages and dendritic cells (DC) to mammalian counterpart that was involved in uptake of foreign antigens [29]. In PBMC, results of cytokines mRNA gene expression revealed that most of tested cytokines markedly elevated early at 6 hpi. That is expected because dendritic cells compared to other antigen presenting cells (macrophage and B cells) can effectively capture and process antigens, express higher level of MHC and co-stimulatory molecules on their surface and also activate naïve T cells [30]. The results of PBMC infection almost equal to that reported from HD11 except for IFNγ gene expression that is higher at variant strain infected cells than that infected with classic strain; nonetheless both viruses grew at a similar level in PBMC. In mammals, the IL4 and IL10 play an important role in the promotion of Th2 responses (humoral immune response) and inhibition of the proinflammatory and Th1 response [31]. This study indicated that after 24hpi skewing toward humoral immune response is essential and differs in HD11 (through IL4) and PBMC (through IL10) that was better marked with variant strain comparing to classical strain. Different expression levels of cytokines between HD11 were previously observed using two different pathotypes of H5N1 [26]. It is worth mentioning that immune response to HPAIV H5N1 varies according the gene constellation of the virus [26] and both Egyptian genotypes have specific genetic markers in all gene segments including the NS1 [6] which is known to be the major viral host-immune system regulator. Thus, the role of internal proteins on different patterns of cytokine expression elicited by both strains in this study should be further elucidated. Generally, innate immune response differs after infection with low pathogenic AIV (LPAIV) and HPAIV [26]. Friesenhagen et al. [32] found that LPAIV induced stronger inflammatory responses in human macrophages than HPAIV which may facilitate wide spread and systemic progression of the later. From various field observations (non published data) variant strain induced lower pathogenicity and delayed onset of death in infected chicken comparing to classical strain infection. Here we investigate the difference in host immune response at the level of immune cells to both strains. Our data revealed that higher innate immune response (IL1b,IL6,IL8 and IFNα) with variant strain infections compared to classical strain infection. These results explain the lower pathogenicity recorded for variant strain may be due to efficient higher innate immune response that in turn skewed to higher humeral immune response (IL4 and IL10). Such mechanism can reduce the variant strain pathogenicity compared to classical strain infection.

5. Conclusion

In conclusion, the immune-escape HPAIV H5N1 elicited higher innate immune response than the "classic" virus. We propose that the capability of the variant virus to induced severe morbidity and mortality among poultry populations across Egypt is probably due to its ability to escape immune response elucidated by vaccination due to antigenic variation. Such variation is thought to affect its epitopes while triggering of higher innate immune response which do not play a significant role in this case. The classic strain produced less cytokine (in magnitudes and types) and it was able to replicate to high titter in HD11 and PMBC, which may indicate adaptation to evade the recognition by the innate immune system in case of infection. Ultimately, there will be two queries in a real need to be investigated; the in-vivo expression of cytokines and the immunogenicity / pathogenicity of the variant strain compared to the classic strain.

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