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Immune-related gene expression in response to H5N1 avian influenza virus infection in chicken and duck embryonic fibroblasts

Qing-long Liang[a,b,1], Jing Luo[a,b,1], Kai Zhou[a,b], Jian-xin Dong[c], Hong-xuan He[a,*]

[a] National Research Center For Wildlife Born Diseases, Key Laboratory of Animal Ecology and Conservation Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China
[b] Graduate University, Chinese Academy of Sciences, Beijing 100049, China
[c] Department of Biology, Chengde Teachers College for Nationalities, Chengde 067000, China

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ABSTRACT

Chicken and ducks are important hosts in responses to highly pathogenic avian influenza virus (HPAIV) H5N1 infection. In ducks, avian influenza (AI) generally causes an asymptomatic and long-lasting infection, whereas clinical apparent and transient disease is often observed in chickens. Using real-time quantitative PCR, we examined the expression of immune-related genes in response to H5N1 infection in chicken embryo fibroblasts (CEF) and duck embryo fibroblasts (DEF). While in CEF IL-6 expressed at high levels similar to mammalian species, in DEF expression levels were minimal. Similarly, duck IFN-β expression were slightly upregulated, whereas chicken expressions were highly upregulated. Chronologically, the mRNA levels of both IFN-alpha and IFN-gamma, which belong to type I and type II interferon, respectively, were unregulated in a similar fashion in chickens than in ducks. IL-2 and TLR-7 were elevated from the beginning of the infection in both CEF and DEF to the end of the experiment. Chicken MHC class I expression was almost unaffected while duck expression were downregulated. DEF and CEF MHC class II expression were downregulated. Chemokine IL-8 expression was upregulated in both species. The IL-8 levels closely parallel the IL-1β induced IL-6 levels in the same samples. These results show distinct embryo fibroblasts expression patterns of pro-inflammatory cytokines and IFNs between species.

1. Introduction

Avian influenza viruses (AIV) are encapsulated by envelopes containing the surface proteins hemagglutinin (HA) and neuraminidase (NA) and are classified by sixteen identified HA subtypes and nine NA subtypes (Fouchier et al., 2005), all of which have been identified in their reservoir hosts such as wild aquatic birds and seabirds (Spackman, 2008; Webster et al., 2006).

The roles of birds as reservoir hosts and hosts in which viruses with pandemic potential could be amplified and transmitted to humans have become a focus of interest with the emergence and perpetuation of H5N1 virus. Despite this, few studies have been done to elucidate basic viral pathogenesis and host response questions in avian species. One of the most interesting questions is that in general, chickens and ducks respond to AIV infections differently and there are many instances demonstrating that infection with a specific AIV isolate might cause lesions and even death in a chicken host, while infection of a duck with the same virus would be asymptomatic, rarely resulting in death (Slemons et al., 1990). AIV shedding in chickens is transient with a rapid clearance by the host (Kwon et al., 2008; Lee et al., 2004). In contrast, intermittent, prolonged shedding is observed in infected ducks (Higgins et al., 1987). While chickens could develop a strong humoral immune response to AIV infection (Suarez and Schultz-Cherry, 2000), it has been reported that ducks do not (Philpott et al., 1989). In addition, though AIV replication has been reported in both the respiratory system and the intestinal tract for both species (Wood et al., 1995), AIV is generally limited in distribution to the intestinal tract in the duck (Scholtissek, 1995), while replication in the upper respiratory tract of chickens with some migration to the intestinal tract is more common (Lee et al., 2007). What's more, influenza virus adapted to efficient growth in ducks, does not always grow in chickens and vice versa and thus, few studies (Adams et al., 2009) have compared viral pathogenesis of or host responses to the same AIV.

The infection of CEF and DEF in vitro with AIV serves as a starting point for observing host responses. By comparing the expression of cytokines involved in pathogen responses including the anti-viral, pro-inflammatory, and cell-mediated and adaptive responses in embryonic fibroblasts, we could further understand how immune responses and thus, pathogenesis might be different between two highly relevant agricultural species. Here we present the results

* Corresponding author. Tel.: +86 10 64807118; fax: +86 10 64807118.
E-mail address: hehx@ioz.ac.cn (H.-x. He).
[1] Qing-long Liang and Jing Luo are co-first authors.

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of quantitative real-time RT-PCR analysis of several cytokines, the TLR-7, and the MHC class I and II molecules expressed in response to infection with the same HPAIV H5N1 in CEF and DEF, which are helpful to fully understand the mechanism underlining the different outcomes of HPAIV H5N1 virus infection in chickens and ducks.

2. Materials and methods

2.1. CEF and DEF culture

CEF and DEF were made by 10-day old chicken embryos and 12-day old Pekin duck embryos (Chinese Academy of Agriculture, Beijing, China). The CEF and DEF were grown overnight in MEM supplemented with 5% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C, 5% CO_2 with a cell density of approximately 1×10^6 cells/100 mm tissue culture dish. After overnight growth, non-adherent cells were removed by washing the monolayers with sterile PBS.

2.2. Viruses and assays

Avian influenza viruses (A/plateau pika/Qinghai/04/2007(H5N1)), subtypes H5N1, were stored in National Research Center for Wildlife Born Diseases, Institute of Zoology. The viruses were grown in the allantoic cavity of 10-day old embryonated chicken eggs (Chinese Academy of Agriculture, Beijing, China) at 37 °C. Allantoic fluid was harvested 48 h after inoculation and stored at −80 °C. Virus titer was measured by hemagglutination (HA) assays. The HA assay was carried out in V-bottom 96-well plates. Serial 2-fold dilutions of virus samples were mixed with an equal volume of a 1% suspension (v/v) of chicken erythrocytes (Chinese Academy of Sciences, Beijing, China) and incubated at room temperature for 30 min. Wells containing an adherent, homogeneous layer of erythrocytes were scored as positive.

2.3. Cell culture and virus infection

Cultures of embryo fibroblasts were made in triplicate for both cells. One embryonic fibroblast culture was trypsinized and the cells were counted. This representative count was used to calculate the volume of virus stock necessary to infect the other three embryonic fibroblast cultures. Embryo fibroblasts were cultured in 100 mm tissue culture dish in MEM for 24 h. The culture medium was then removed and 100 μl of H5N1 virus [5000 plaque-forming units (pfu)/mL] in infection medium, consisting of MEM, 5% heat-inactivated fetal bovine serum, was added to each dish. Negative control embryo fibroblast cultures were set up identically but without the addition of virus. Positive control embryo fibroblast cultures were set up identically with the addition of lipopolysaccharide (LPS, 10 ng/ml). Culture plates were gently rocked every 15 min for 1 h after which the media was replaced with MEM supplemented with 1% heat-inactivated fetal bovine serum. Cultures were incubated and RNA extracted from the cell monolayer at 8, 24, and 36 h post-infection (hpi).

2.4. Hemagglutination assay

Virus titer was measured by hemagglutination assay. The hemagglutination assay was carried out in V-bottom 96-well plates. Serial 2-fold dilutions of Supernatants (50 μl) from CEF and DEF were mixed with an equal volume of a 1% suspension (v/v) of chicken erythrocytes and incubated at room temperature for 30 min. Wells containing an adherent, homogeneous layer of erythrocytes were scored as positive.

2.5. RNA and cDNA preparation

DNase-treated total RNA was isolated by using RNApure® total RNA extraction kit according to the protocol of the manufacturer (Bioteke, China). RNA in each sample was quantified using Ultrospec 2000 mass spectrophotometer. Reverse transcription (RT) was carried out by using an Omniscript reverse transcriptase kit (Qiagen, Germany) in a 25-μl reaction mixture, containing 2 μg of total RNA and primers of oligo(dT)15, at 42 °C for 1 h.

2.6. Quantitative real-time PCR

qRT-PCR was performed using primers designed by Primer Premier5.0 software based on published target sequences and previously reported (Adams et al., 2009). Primers were developed for IL-1β, IL-2, IL-6, interferon alpha (IFN-α), interferon beta (IFN-β), interferon gamma (IFN-γ), TLR-7, MHC class I, and MHC class II and IL-8 molecules based on published sequences and the predicted product sizes are shown in Table 1. Primers pairs were selected based on specificity as determined by dissociation curves. The levels of PCR products were monitored with the Stratagene Mx3005 using SYBER Green PCR Master Mix (TaKaRa, China). PCR conditions were the same for each targeted gene and are as follows: 95 °C for 30 s, followed by 45 cycles of 95 °C for 15 s, 58 °C for 20 s and 72 °C for 20 s. Cycling was terminated after 45 cycles with 95 °C for 1 min, 60 °C for 1 min, and 95 °C for 30 s. Dissociation curves of the products were generated by increasing the temperature of samples incrementally from 55 to 100 °C as the final step of the real-time PCR. Amplified products were run on a gel and extracted using a PCR and Gel Purification kit (TianGen, China). For the purpose of assay validation, purified products were cloned into pMD18-T with a TA Cloning kit (TaKaRa, China) and sequenced to verify proper target amplification using M13 forward and reverse primers.

2.7. Calculations and statistics

The house keeping gene glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was used as an internal control, and quantification of the transcripts was performed by the $-\Delta\Delta C_t$ method (Livak and Schmittgen, 2001). Each subsequent time point (8, 24, 36 hpi) compared against baseline ($t = 0$ hpi) transcript level. Logarithmic transformation was performed on fold change values before being analyzed by the Student's *T*-test (Microsoft Excel 2007). The Student's *T*-test was used to determine significant difference between fold change values of CEF and DEF transcripts. Standard error was calculated using the fold change values of three replicates for each gene measured.

3. Results

3.1. Hemagglutination assay of infected DEF and CEF

At different times after infection, culture supernatants were harvested, serially diluted, and assayed to determine the virus titer by using an HA assay. As shown in Table 3, at 8 hpi, the virus titers were too low to detect. Then the virus titers increased to 2^6 and 2^3 at 24 hpi in CEF and DEF, respectively. Similarly, at 36 hpi, the virus titers maintained at a similar level with 24 hpi. To sum up, the virus titer in DEF was lower than in CEF in the whole experiment.

3.2. Differential expression of pro-inflammatory, anti-viral, and Th1-associated cytokines in DEF and CEF infected with H5N1 influenza virus

The expression of pro-inflammatory cytokines IL-1β were similar in CEF and DEF at the early stage of viral infection, with a low

Table 1
Primer sequences used in the study.

Primer name	Sequence (5′ → 3′)	Product size (bp)	Reference
CGAPDHF	CCTCTCTGGCAAAGTCCAAG	200	V00407
CGAPDHR	CATCTGCCCATTTGATGTTG		
CIL-1βF	GCTCTACATGTCGTGTGTGATGAG	80	NM204524
CIL-1βR	TGTCGATGTCCCGCATGA		
CIL-2F	CGGGATCCATGATGTGCAAAGTACTG	80	AY510091
CIL-2R	CGGTCGACTTATTTTTGCAGATATCT		
CIL-6F	ATGTGCAAGAAGTTCACCGTG	171	EU170468
CIL-6R	TTCCAGGTAGGTCTGAAAGGCGAA		
CIFNαF	ATGCCACCTTCTCTCACGAC	387	EU367971
CIFNαR	AGGCGCTGTAATCGTTGTCT		
CIFNγF	GCTGACGGTGGACCTATTATT	248	DQ906156
CIFNγR	TGGATTCTCAAGTCGCTCATCG		
CMHC IF	AAGAAGGGGAAGGGCTACAA	222	NM001031338
CMHC IR	AAGCAGTGCAGGCAAAGAAT		
CMHC IIF	CTCGAGGTCATGATCAGCAA	312	DQ008588
CMHC IIR	TGTAAACGTCTCCCCTTTGG		
CTLR-7F	TGTGATGTGGAAGCCTTTGA	218	DQ780342
CTLR-7R	ATTATCTTTGGGCCCCAGTC		
DGAPDHF	ATGTTCGTGATGGGTGTGAA	176	AY436595
DGAPDHR	CTGTCTTCGTGTGTGGCTGT		
DIL-1βF	TCGACATCAACCAGAAGTGC	185	DQ393268
DIL-1βR	GAGCTTGTAGCCCTTGATGC		
DIL-2F	GCCAAGAGCTGACCAACTTC	137	AF294323
DIL-2R	ATCGCCCACACTAAGAGCAT		
DIL-6F	TTCGACGAGGAGAAATGCTT	150	AB191038
DIL-6R	CCTTATCGTCGTTGCCAGAT		
DIFNαF	TCCTCCAACACCTCTTCGAC	232	EF053034
DIFNαR	GGGCTGTAGGTGTGGTTCTG		
DIFNγF	GCTGATGGCAATCCTGTTTT	247	AJ012254
DIFNγR	GGATTTTCAAGCCAGTCAGC		
DMHCIF	GAAGGAAGAGACTTCATTGCCTTGG	196	AB115246
DMHC IR	CTCTCCTCTCCAGTACGTCCTTCC		
DMHC IIF	CCACCTTTACCAGCTTCGAG	229	AY905539
DMHC IIR	CCGTTCTTCATCCAGGTGAT		
DTLR-7F	CCTTTCCCAGAGAGCATTCA	154	AY940195
DTLR-7R	TCAAGAAATATCAAGATAATCACATCA		
IFNβF	CCTCAACCAGATCCAGCATT	259	AY831397
IFNβR	GGATGAGGCTGTGAGAGGAG		
IL-8F	AAGTTCATCCACCCTAAATC	182	NM205498
IL-8R	GCATCAGAATTGAGCTGAGC		

expression level of 0.32 fold and 0.42 fold at 8 hpi and then a slight increase of 1.44 fold and 1.24 fold at 24 hpi, respectively, while it is statistically different (at $p < 0.05$) at 36 hpi for CEF and DEF (0.94 fold and 2.18 fold, respectively) (Fig. 1 and Table 2). Dissimilarly, IL-6 expression was upregulated to 2.95 fold and 3.35 fold in CEF at 8 and 24 hpi, respectively, and a little down to 1.42 fold at 36 hpi (Fig. 1 and Table 2). However, it was nearly unchanged at 8 hpi (0.92 fold) and maintained at 1.65 and 1.48 fold at 24 and 36 hpi, respectively. IL-8 expression was gradual upregulated from baseline level at 8 hpi to the high level at 24 and 36 hpi, with a higher level in DEF compared to CEF at all time points (Fig. 1 and Table 2). Differently, the peak occurs at 24 hpi (12.47 fold at 24 hpi and 10.56 fold at 36 hpi) in DEF while in CEF IL-8 expressed a little weaker at 24 hpi (4.72 fold) than 36 hpi (5.39 fold).

We compared type I and II IFN expression in CEF and DEF infected with H5N1 and observed a similar species dependent response. The pattern of IFN-α response in chicken and duck were similar with peak expression occurring at 24 hpi and maintained till 36 hpi (Fig. 2 and Table 2). The differences in expression levels for IFN-α were statistically different ($p < 0.01$) between DEF and CEF. The comparative expression of IFN-β represents the most striking finding in this study: chicken IFN-β is highly expressed and peaks at 1237 fold expression while the duck response is never more than 3.3 fold elevated (36 hpi). IFN-γ is induced at clearly different expression level despite insignificantly difference ($p > 0.05$): 421 fold for CEF and 69 fold for DEF by 24 hpi and 538 fold for CEF and 72 fold for DEF by 36 hpi, Taken together, these results indicate that the IFN signaling in DEF is weaker than the extremely robust expression of IFN observed in CEF.

The expression of the Th1-involved cytokine, IL-2, followed the same pattern of gradual increase at 24 and 36 hpi, but a little lower in CEF than DEF: 27 and 100 fold in CEF while 64 and 132 in DEF by 24 and 36 hpi, respectively (Fig. 1 and Table 2).

3.3. Late induction of TLR-7 expression in DEF and lasting induction in CEF

We compared the expression of TLR-7 in response to infection with H5N1 in CEF and DEF. The results showed that in chicken TLR-7 messages was induced by 8 hpi (1.74 fold) and peaked at 36 hpi (3.16 fold), while in duck it was induced slightly at 36 hpi (2.87 fold) (Fig. 3 and Table 2). Despite the lack of statistically significant differences at the 8 and 24 hpi points, the trend of TLR-7 expression is clearly different: DEF express elevated levels (2.87 fold induction) of TLR-7 late during the infection. In contrast, CEF expression of TLR-7 was gradually induced from baseline levels at 8 hpi to a 3.16 fold induction by 36 hpi.

3.4. Downregulated MHC class I and II molecule expression

MHC class I and II molecule expression was downregulated in CEF and DEF at all time points except 8 hpi in CEF (Fig. 4 and Table 2). By 8 hpi, MHC class I was upregulated 1.19 fold in CEF and downregulated 0.45 in DEF with a significant statistical different

Table 2
Differential genes expression in CEF and DEF after infected with H5N1 influenza virus and LPS.

Gene	Cells	Time	H5N1 influenza virus			LPS		
			Fold changes	Range up	Range low	Fold changes	Range up	Range low
IL-1β	CEF	8	0.32	0.41	0.25	2.36	2.50	2.16
	CEF	24	1.44	2.06	1.01	0.97	1.16	0.86
	CEF	36	0.94	1.19	0.74	1.69	1.99	1.51
	CEF	NC	1.00	1.28	0.78	1.00	1.00	1.00
	DEF	8	0.42	0.57	0.31	27.47	48.50	16.91
	DEF	24	1.24	1.60	0.97	2.36	3.41	1.37
	DEF	36	2.18	2.97	1.61	2.07	2.64	1.66
	DEF	NC	1.00	1.41	0.71	1.00	1.00	1.00
IL-2	CEF	8	0.79	1.76	0.36	/	/	/
	CEF	24	27.54	39.89	19.01	/	/	/
	CEF	36	100.89	173.34	58.72	/	/	/
	CEF	NC	1.00	1.62	0.62	/	/	/
	DEF	8	1.25	3.55	0.44	3.56	7.21	1.93
	DEF	24	64.15	85.64	48.05	0.65	1.41	0.25
	DEF	36	132.51	175.36	100.14	1.39	3.97	0.75
	DEF	NC	1.00	1.42	0.70	1.00	1.00	1.00
IL-6	CEF	8	2.95	4.12	2.11	0.99	1.04	0.90
	CEF	24	3.35	4.91	2.28	0.67	0.71	0.64
	CEF	36	1.42	2.13	0.95	0.98	1.04	0.86
	CEF	NC	1.00	1.59	0.63	1.00	1.00	1.00
	DEF	8	0.92	1.58	0.54	15.82	21.56	11.31
	DEF	24	1.65	2.36	1.16	3.57	4.03	3.01
	DEF	36	1.48	1.92	1.14	1.79	1.93	1.60
	DEF	NC	1.00	1.36	0.74	1.00	1.00	1.00
IL-8	CEF	8	2.16	7.16	0.65	9.87	10.41	9.13
	CEF	24	4.72	12.91	1.73	3.15	4.47	2.46
	CEF	36	5.39	15.64	1.86	30.98	149.09	13.27
	CEF	NC	1.00	2.78	0.36	1.00	1.00	1.00
	DEF	8	3.95	9.80	1.59	29.11	37.53	19.56
	DEF	24	12.47	28.60	5.43	10.34	12.30	8.69
	DEF	36	10.56	28.06	3.97	3.78	4.56	
	DEF	NC	1.00	2.95	0.34	1.00	1.00	1.00
IFN-α	CEF	8	7.31	10.24	5.22	0.19	0.26	0.13
	CEF	24	556.41	705.35	438.92	0.08	0.09	0.07
	CEF	36	310.83	404.70	238.74	0.67	0.86	0.43
	CEF	NC	1.00	1.37	0.73	1.00	1.00	1.00
	DEF	8	1.41	1.76	1.12	3.52	4.59	2.83
	DEF	24	46.31	54.89	39.08	0.57	1.21	0.25
	DEF	36	39.49	46.57	33.48	1.33	2.85	0.39
	DEF	NC	1.00	1.20	0.83	1.00	1.00	1.00
IFN-β	CEF	8	53.57	98.56	29.12	0.19	0.20	0.17
	CEF	24	980.02	1808.93	530.94	0.08	0.11	0.06
	CEF	36	1237.60	2289.24	669.07	0.91	1.09	0.78
	CEF	NC	1.00	2.35	0.43	1.00	1.00	1.00
	DEF	8	0.71	2.55	0.20	1.05	9.13	0.15
	DEF	24	2.98	6.77	1.31	0.40	1.24	0.16
	DEF	36	3.27	7.04	1.52	0.37	7.67	0.02
	DEF	NC	1.00	2.69	0.37	1.00	1.00	1.00
IFN-γ	CEF	8	14.22	29.12	6.95	/	/	/
	CEF	24	421.68	580.98	306.06	/	/	/
	CEF	36	538.70	807.69	359.29	/	/	/
	CEF	NC	1.00	1.30	0.77	/	/	/
	DEF	8	1.11	3.29	0.37	/	/	/
	DEF	24	69.23	206.28	23.23	/	/	/
	DEF	36	72.00	237.94	21.79	/	/	/
	DEF	NC	1.00	4.60	0.22	/	/	/
MHCI	CEF	8	1.19	1.37	1.03	0.67	0.82	0.60
	CEF	24	0.69	0.79	0.61	0.94	1.22	0.75
	CEF	36	0.43	0.47	0.39	0.88	0.99	0.76
	CEF	NC	1.00	1.14	0.88	1.00	1.00	1.00
	DEF	8	0.45	0.49	0.41	1.23	1.38	0.97
	DEF	24	0.75	0.84	0.66	1.18	1.67	0.88
	DEF	36	0.59	0.71	0.49	1.11	1.14	1.05
	DEF	NC	1.00	1.11	0.90	1.00	1.00	1.00
MHCII	CEF	8	0.21	0.37	0.12	/	/	/
	CEF	24	0.55	0.94	0.32	/	/	/
	CEF	36	0.35	1.31	0.09	0.06	0.18	0.02
	CEF	NC	1.00	2.00	0.50	1.00	1.00	1.00
	DEF	8	0.41	0.46	0.36	0.29	0.34	0.25
	DEF	24	0.08	0.11	0.06	0.15	0.20	0.12

Table 2 (*Continued*)

Gene	Cells	Time	H5N1 influenza virus			LPS		
			Fold changes	Range up	Range low	Fold changes	Range up	Range low
TLR7	DEF	36	0.08	0.16	0.04	0.15	0.22	0.11
	DEF	NC	1.00	1.07	0.94	1.00	1.00	1.00
	CEF	8	1.74	2.06	1.47	0.77	1.93	0.40
	CEF	24	1.84	2.68	1.27	0.41	1.21	0.20
	CEF	36	3.16	4.37	2.28	1.80	3.20	1.24
	CEF	NC	1.00	1.25	0.80	1.00	1.00	1.00
	DEF	8	0.87	3.02	0.25	4.40	8.63	2.91
	DEF	24	1.04	2.61	0.42	1.00	1.75	0.33
	DEF	36	2.87	4.72	1.74	4.24	19.29	1.45
	DEF	NC	1.00	1.96	0.51	1.00	1.00	1.00

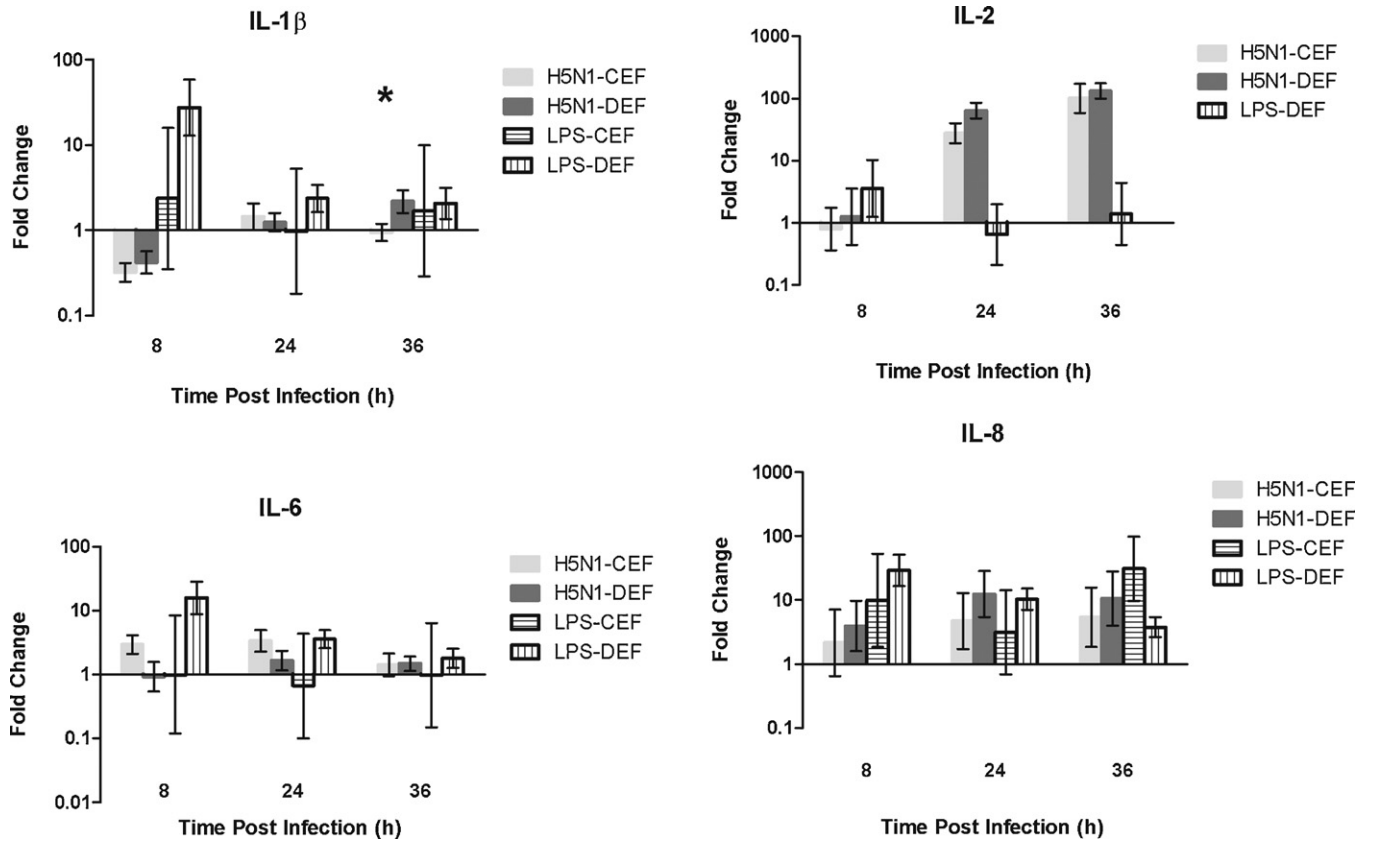

Fig. 1. Cytokine expression in DEF and CEF in response to H5N1 infection: fold change expression of IL-1β, IL-2, IL-6 and IL-8. (*) Indicates $p < 0.05$ for the comparison of DEF and CEF transcripts as determined by the Student's *T*-test. Error bars represent standard error.

Table 3
HA test of CEF and DEF after challenged by H5N1.

Cell name	8 hpi	24 hpi	36 hpi
CEF	0	2^6	2^6
DEF	0	2^3	2^4

($p < 0.01$), whereas MHC class II molecules were downregulated in both species by 0.21 fold in CEF and 0.41 fold in DEF. In CEF and DEF, MHC class I molecules were expressed at nearly identical levels at 24 and 36 hpi, whereas MHC class II molecules were statistically different ($p < 0.05$) at 24 hpi.

4. Discussion

Primary CEF and DEF are readily used to study influenza infection because of their high susceptibility, thus are suitable to detect changes in gene expression very early in the course of infection under controlled conditions by real time RT-PCR. As shown here, this model was used to directly compare the effects of influenza virus infection in chickens and ducks.

More recently, it has been shown that respiratory epithelial cells and different leukocyte populations elaborate biologically active cytokines, including interleukin (IL)-1, tumor necrosis factor-α, IL-6, and IL-8, when exposed to or infected with influenza virus (Matsukura et al., 1996). These cytokines are shown to provoke symptoms and signs consistent with those of a viral upper respiratory tract infection when administered systemically or locally (Douglass et al., 1994; Emery et al., 1992; Vial and Descotes, 1994). The results of our study indicate that the expression level of the pro-inflammatory factors such as IL-1β, IL-2, IL-6 and IL-8, the IFNs, pattern recognition receptor such as TLR-7, MHC, are distinct in the two species. In this study the positive control treatment with LPS shows that species differences are specific to H5N1 and not inherent differences in the fibroblasts ability to make certain cytokines. So, we could compare several fibroblast cell lines to ensure

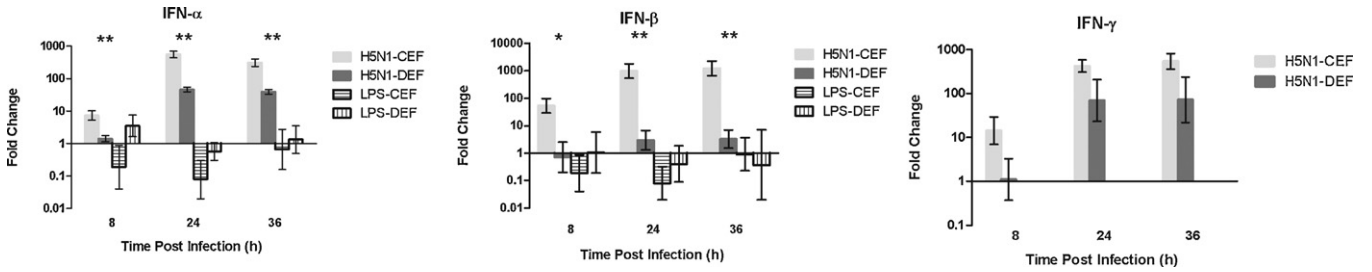

Fig. 2. Interferon expression in DEF and CEF in response to H5N1 infection. (*) indicates $p < 0.05$ and (**) indicates $p < 0.01$ for the comparison of DEF and CEF transcripts as determined by the Student's *T*-test. Error bars represent standard error.

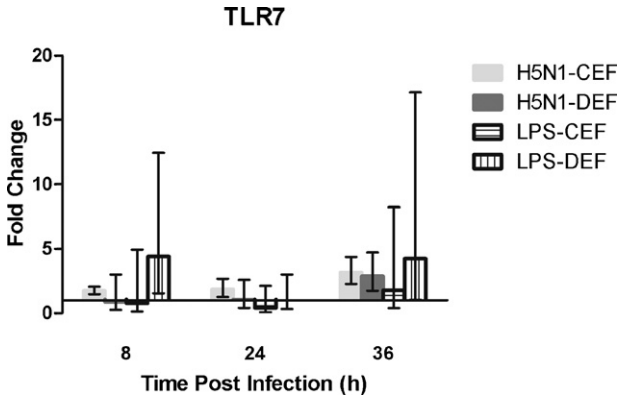

Fig. 3. Fold change expression of TLR-7 by DEF and CEF in response to H5N1 infection.

our observations are not artifacts of the particular cells we are using.

In one study, data supported a causal relationship between viral replication, cytokine production, and symptom expression, and they suggested that IL-6 may have a role in mediating symptom and sign expression during influenza A virus infection (Skoner et al., 1999). Here, the result that the induction of IL-6 in chickens is more robust than ducks may be related to the differences signs after influenza virus infection in the two species. In contrast, IL-8 expressed more greatly in ducks than chickens, which is similar with the results of the 1918 influenza virus infection of macaques (Kobasa1 et al., 2007). It was also reported that the elevation of IL-2 may have the protect effect against influenza virus (Henke et al., 2006). This is consistent with the results that IL-2 expressed more in DEF.

Comparing the expression levels of both type I IFNs, CEF expresses higher levels of both IFN-α and IFN-β than DEF. A weak induction of IFN has been correlated in chickens with higher virus titers and a longer shedding period, whereas strong IFN expression results in lower virus titers and a shorter shedding period (Cauthen et al., 2007). Based on these findings, we conclude that the lower overall expression of IFNs by DEF in response to AIV infection reflect what happens at the organismal level, longer shedding and weaker viral clearance observed in duck, and more rapid clearance and thus a relatively shorter shedding period in chicken.

TLR7 is triggered by antiviral compounds and single-stranded RNA, and is implicated in the immune response to viruses such as influenza. TLR7 expression in lung, and upregulation of IFN-α by TLR7 agonists, not typically seen in chickens, could contribute significantly to the antiviral defense of ducks. These differences in TLR7 function, not genomic organization, may contribute to the differential susceptibility of avian species to viral infection. Duck TLR7 was highly expressed in lymphoid tissues such as the spleen and bursa (MacDonald et al., 2008). In addition, high expression is seen in the lung tissue of ducks, which is distinct from the expression pattern of chickens. The duck TLR7 expression pattern is comparable to that for human, which is highest in spleen, with significant expression in lung (Chuang and Ulevitch, 2000; Nishimura and Naito, 2005). High pulmonary expression of TLR7 could be significant in the context of highly pathogenic H5N1 avian influenza, which is primarily a lung infection (Pantin-Jackwood and Swayne, 2007). The observed difference in TLR7 expression may be due to differences in the organization of lymphoid tissue in the lung of ducks and chickens, or the presence of resident cells expressing the TLR7 receptor, which are absent in the chicken (MacDonald et al., 2008).

Studies have demonstrated that viruses have evolved mechanisms to inhibit MHC class I expression by interfering with the function of the MHC class I assembly machinery in the endoplasmic reticulum and by exploiting endoplasmic-reticulum-associated degradation pathways (Yewdell and Bennink, 1999). In our study,

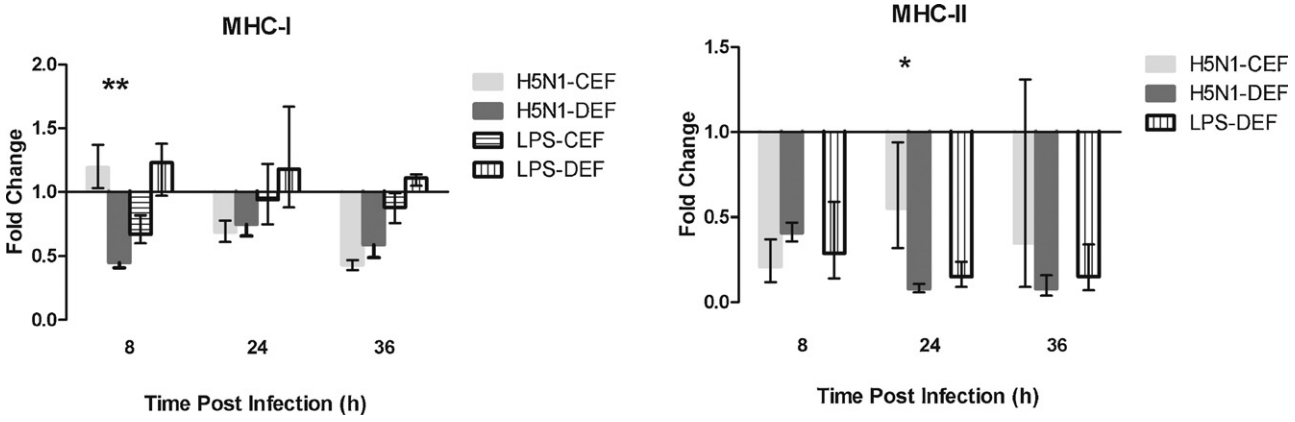

Fig. 4. Fold change expression of MHC by DEF and CEF in response to H5N1 infection. (*) Indicates $p < 0.05$ and (**) indicates $p < 0.01$ for the comparison of DEF and CEF transcripts as determined by the Student's *T*-test. Error bars represent standard error.

repression of MHC class I and II mRNA expression by infection with virus was not surprising, because it has been shown that the ability of influenza viruses to modulate the mRNA expression of MHC class I varies. In one study, influenza A virus (H3N2) up-regulated MHC class I mRNA expression levels (Tong et al., 2004). In another study, the expression of MHC class I did not increase due to infection of macrophages with a low pathogenic H7N2 AIV (Keller et al., 2007). Both studies may explain the unusual upregulation of MHC class I mRNA in chickens at 8 hpi.

In our study, DEF and CEF were challenged by the same quantity of virus and the titer in DEF was lower than in CEF throughout the whole experiment. It can be inferred that the rates of viral entry and/or replication are occurring differently between the two species. In order to make it clarify whether different virulence of virus and other types of cells (e.g. respiratory epithelial cells) have different patterns of cytokines induction additional experiments will be needed for the further study.

Avian influenza virus can infect a variety of birds and mammals. Its natural hosts are ducks, gulls, shorebirds and other waterfowl, while the Galliformes, primarily chickens and turkeys, and mammalian species represent an abnormal host for influenza infection (Suarez and Schultz-Cherry, 2000). The ability to cause disease and the ability of the host to respond to influenza varies greatly by species. The pathogenesis of avian influenza in different species can also be very different, primarily when comparing ducks to chickens and turkeys. Here we compare transcript levels for a variety of cytokines and immune-associated in DEF and CEF following in vitro challenge with H5N1 avian influenza virus. We attempt to provide a comprehensive comparison of innate, intrinsic immunity against H5N1. On the surface, the information would be useful and should provide insight into pathogenesis of the virus due to host facts. However, the detailed mechanism of these questions still remain further discussed.

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