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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