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Research paper

Differential cellular immune responses between chickens and ducks to H9N2 avian influenza virus infection

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ABSTRACT

Avian influenza is an important infectious disease for the poultry industry and an ongoing public health concern. In this study, monoclonal antibodies (mAbs) specific to duck CD3ε, CD4 and CD8α were generated by immunizing mice with the corresponding *Escherichia coli*-expressed proteins and producing hybridomas. The resulting mAbs were used to investigate cellular immune responses of ducks and chickens during H9N2 avian influenza A virus (AIV) infection. By flow cytometric analysis, responses of T lymphocytes, especially $CD8^+$, $CD8^+CD25^+$ and $CD4^+CD25^+$ T cells, were stronger in ducks than in chickens following H9N2 AIV-infection. By quantitative real-time PCR analysis, virus mRNA could be detected in cloaca and oropharynx from both bird species and in spleens from chickens, and distinctive kinetics of transcriptional levels of *interleukins* and *interferons* were exhibited between chickens and ducks. With ducks showing more active and robust cellular immune responses than chickens, these results revealed that the distinct responses to H9N2 AIV infection may contribute to the different susceptibilities to AIV infection between the two species.

1. Introduction

Avian influenza is an infectious disease that significantly impacts the poultry industry and public health. Following influenza virus infection, the innate immune response is the first defense against invading pathogens and is critical to viral clearance. However, adaptive cellular immune responses are also protective against influenza, and lymphocytes play an important role in host responses to infection. Conventional $CD4^+$ T cells and $CD8^+$ T cells, defined as helper T lymphocyte (Th) and cytotoxic T lymphocyte respectively, are both important in the clearance of intracellular pathogens such as viruses, certain bacteria and tumors (Alexander-Miller, 2005; Kaech et al., 2002; Wherry and Ahmed, 2004). It has been shown that cloned influenza-specific $CD8^+$ T cells can passively transfer protection against infection (Taylor and Askonas, 1986). Adoptive transfer of $CD8^+$ T cells from H9N2 subtype avian influenza virus (AIV)-infected chickens to naïve chickens can protect them from challenge with lethal H5N1 AIV, suggesting the presence of cross-reactive cellular immunity between AIV-infected and naïve chickens, and virus clearance by $CD8^+$ T cells (Seo and Webster, 2001). $CD4^+$ T cells contribute to the development of secondary and memory responses by $CD8^+$ T cells (Belz et al., 2002; Brooks et al., 1999), but not primary responses (Belz et al., 2003). Additionally, $CD25^+$ cells that may play a regulatory role in the immune response have been shown to be transiently upregulated in H9N2 AIV-infected chickens and ducks (Teng et al., 2006; Wang et al., 2007). Our previous data also showed a correlation between kinetics of $CD25^+$ cells in peripheral blood mononuclear cells (PBMC) and

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serum soluble CD25 concentrations with disease severity during duck AIV or bacterial infection (Huang et al., 2011).

Recent reports showed that mRNA encoding interferon (IFN)-γ is upregulated in H9N2 AIV-infected chickens, while those for major histocompatibility complex antigens II, interleukin (IL)-4, IL-4 receptor and CD74 are downregulated in the lung, all of which are pivotal to the activation of $CD4^+$ helper T cells and humoral immunity (Xing et al., 2008). Karpala et al. (2011) also showed that inflammatory Th1, not Th2, cytokines are induced by highly pathogenic H5N1 AIV in chickens.

The above-mentioned results were mainly obtained from chickens, the AIV natural host. Ducks are usually asymptomatic and exhibit long-term viral shedding after AIV infection, which make them "Trojan horses" in the spread of AIV (Kim et al., 2009). *In vitro*, distinct expression patterns of immune-related genes have been found in PBMC (Adams et al., 2009) and embryo fibroblasts (Liang et al., 2011) between chickens and ducks following low and high pathogenic AIV infection. However, the information derived from ducks as a natural reservoir host of all known AIV subtypes (Olsen et al., 2006) is still limited due to lack of reagents and poorly conserved genes between chickens and ducks (Yuan et al., 2005). Even in the same species, monoclonal antibodies (mAbs) against Pekin duck antigens do not react with those of the Muscovy duck, suggesting some epitopes are not conserved between these two species (Kothlow et al., 2005). Until now, differences between these two species in cellular immune responses to AIV infection have been largely unknown. Here, by generating mAbs to duck CD molecules, we were able to analyze the cellular immunity in ducks and chickens using the H9N2 virus as a model. The results showed differential cellular immune responses to H9N2 AIV infection between chickens and ducks.

2. Materials and methods

2.1. Animals and virus

Specific pathogen free (SPF) Leghorn chickens, Muscovy ducks (*Cairina moschata*), Pekin ducks and Shaoxing ducks were purchased from the Yuyao Shennong Poultry Co., Ltd., Zhejiang Province of China and raised in high-efficiency particulate air-filtered negative-pressure isolators with *ad libitum* access to feed during the experimental stage. BALB/c mice were purchased from the Shanghai Laboratory Animal Center, Chinese Academy of Sciences, Shanghai, China. AIV A/Ck/JD/323/2001 (H9N2 virus, $10^{6.5}$ ELD_{50}/0.1 ml) was stored in our laboratory (Wang et al., 2007). All experimental protocols involving animals have been approved by the Scientific Ethical Committee of Zhejiang University, China.

2.2. Cloning and prokaryotic expression of duck CD molecules

Total cellular RNA was extracted from splenocytes collected aseptically from a 35-day-old Muscovy duck as previously described (Zhou et al., 2005) with Trizol reagent (Invitrogen, USA) and reverse-transcribed into cDNA using a universal reverse primer oligo$(dT)_{18}$. PCR was performed to amplify the coding regions of the duck CD3ε (dCD3ε), CD4 (dCD4) and CD8α (dCD8α) extracellular domain using primers shown in Table 1. To identify the homology of these CD molecules among Muscovy, Pekin and Shaoxing ducks, PCR was also performed using the same primers. The purified PCR products of *dCD3ε* and *dCD8α* were cloned into the vectors pET32a and pET28a (Novagen, USA), respectively. The PCR product of *dCD4* was introduced into the plasmid pET28a. The recombinant plasmids were then transformed into competent *Escherichia coli* BL21 (DE3) cells (Invitrogen). Finally, each recombinant protein bearing a poly-histidine tag was expressed by isopropyl-β-D-thiogalactoside induction and purified by nickel column chromatography (Qiagen Inc., USA). The products were identified by SDS-PAGE and Western blot with the anti-His mAb (Amersham, USA) as described previously (Zhou et al., 2005).

2.3. Generation, identification and labeling of mAbs

The mAbs to *E. coli*-expressed dCD3ε, dCD4 and dCD8α ectodomain were generated by standard procedures. Briefly, 6-week-old BALB/c mice were immunized three times with 75 μg recombinant dCD3ε (rdCD3ε), recombinant dCD4 (rdCD4) or recombinant dCD8α (rdCD8α) protein in complete Freund's adjuvant (Sigma, USA) intraperitoneally at 3-week intervals, followed by one boost with 150 μg proteins. Mouse spleen cells were fused with Sp2/0 myeloma cells *in vitro* three days after the protein boost. Hybridoma cells were screened for specificity to rdCD3ε, rdCD4 and rdCD8α by indirect ELISA and confirmed by Western blot as described earlier (Teng et al., 2006; Wang et al., 2007).

To screen for mAbs capable of recognizing endogenous dCD3ε, dCD4 and dCD8α molecules, an indirect immunofluorescence assay was performed as described previously (Teng et al., 2006; Wang et al., 2007). In brief, blood was collected from adult Muscovy ducks with an equal volume of Alsever's solution (0.42% NaCl, 0.8% trisodium citrate, 2.05% glucose; adjusted to pH 6.1 with 10% citric acid solution). PBMC (1×10^6) isolated by using Histopaque 1077 (Sigma, USA) were blocked by PBS containing 5% normal mouse serum for 30 min on ice and then incubated with the mAbs to dCD3ε, dCD4 or dCD8α, followed by FITC-labeled goat anti-mouse-IgG (SBA, USA). Finally, to localize the expressed dCD3ε, dCD4 and dCD8α molecules, further examination was performed by confocal laser scanning microscopy (Carl Zeiss MicroImaging Inc., Germany). Samples incubated with 5% normal mouse serum and FITC-labeled goat anti-mouse-IgG were used as negative controls.

In order to detect the reactivity of mAbs with endogenous proteins, PBMC and splenocytes were isolated from Muscovy ducks. Cell extracts were prepared in radio immunoprecipitation assay buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM Na_3VO_4, 1 mM EDTA, 1% Triton X-100 and 1 mM phenylmethanesulfonyl fluoride. After centrifuging the samples at 4 °C (12,000 rpm) for 5 min, the supernatants were collected and analyzed by Western blot.