Molecular Immunology 76 (2016) 62–69

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Recombinant chicken interferon-alpha inhibits the replication of exogenous avian leukosis virus (ALV) in DF-1 cells

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ARTICLE INFO

Article history:
Received 15 March 2016
Received in revised form 14 June 2016
Accepted 17 June 2016

Keywords:
Exogenous avian leukosis virus
Chicken alpha interferon
Virus replication
Cytokines
IFN regulatory factor 3

ABSTRACT

Chickeninterferon alpha (ChIFNα) belongs to type I IFNs that are important antiviral cytokines. We investigated whether ChIFNα plays a role in avian leukosis virus (ALV) infections of chickens. Firstly, we explored the immune response to ALV *in vivo* by measuring cytokine expression profiles in the spleens and bursas of chickens during the late stages of ALV-J infection. The results indicated that ALV-J infection could induce a mixed Th1/Th2 cytokine response by elevating levels of both interleukin-2 (IL-2) and IL-10. In contrast, tumor necrosis factor alpha (TNF-α) levels decreased in the spleen while interferon beta (IFNβ) and Toll-like receptor 7 (TLR7) expression levels in the bursa increased significantly. This indicated that ALV-J stimulates a Type I IFN response. Next, we found that different ALV subgroups or strains up-regulated chicken IFN regulatory factor 3 (ChIRF-3) promoter activity, suggesting that ALV infection could trigger Type I IFNs pathway *in vitro*. Accordingly, we further investigated ChIFNα antiviral effects on ALV replication in DF-1 cells by successfully expressing recombinant ChIFNα in *Escherichia coli* (*E. coli*) strain BL21. The specific activity of the purified rChIFNα protein was determined to be 4×10^7 U/mL. When added at 4000 U/mL, the recombinant protein restrained ALV replication as measured by decreases in viral protein p27 levels and mRNA expression. This new reagent may be useful for prophylactic and therapeutic drug design.

1. Introduction

Avian leucosis virus (ALV) is a chicken retrovirus that causes significant economic losses in the poultry industry. In poultry flocks, overt mortality and eradication costs as well as decreased productivity due to tumor production are increasing worldwide. It is therefore a global concern in the poultry industry.

ALV was the first oncogenic virus discovered more than a century ago and has been extensively studied as a retroviral model. ALV subgroups A, B, and J are the most common pathogenic types and are classified according to their viral envelope glycoproteins. ALV subgroups A (ALV-A) and B (ALV-B) mainly induce the lymphoid leukosis (LL) or erythroblastosis (EB) in susceptible chickens (Tam et al., 2002). But beyond that, ALV subgroups J (ALV-J) is also the main cause of myeloid leukosis (ML) and hemangioma (Li et al., 2013). ALV transmission from an infected hen occurs by virus shedding into the egg albumin or yolk and horizontal transmission can occur *via* ingestion of contaminated feces from a viremic hen. This further complicates prevention and control of ALV infection in chicken flocks. To date, the use of vaccines to control the disease has not been successful. This is at least partially due to the immunological tolerance of chickens, the high ALV mutation rate and host factors involved in viral infection (Payne and Nair, 2012; Qian et al., 2014; Wang et al., 2011).

Abbreviations: ALV, avian leukosis virus; ALV-A/B/J, avian leukosis virus subgroup A/B/J; ChIFNα, chicken interferon alpha; ChIRF-3, chicken IFN regulatory factor 3.

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http://dx.doi.org/10.1016/j.molimm.2016.06.012
0161-5890/

Cytokines play key roles in immune response regulation and are essential for host defenses against viral, bacterial and protozoan pathogens as well as to tumors (Belardelli, 1995; Read and Douglas, 2014; Wang et al., 2014). Type I interferons (IFN) are cytokines that direct both innate and adaptive immune responses to viral infections. Chickens produce two type I interferons (α and β) and ChIFNα has stronger antiviral activity *in vitro* which is attributed to the greater expression levels of downstream antiviral IFN-stimulated genes (Qu et al., 2013). Type I IFN expression is controlled by latent transcription factors including IFN regulatory factor 3 (IRF-3) (Martinez-Sobrido et al., 2006). Interestingly, the structure of the chicken IRF-3 orthologue is unique and unrelated to other metazoan orthologues although its function is similar. However, few studies have been conducted to address what type of immune responses the late stage of ALV infection triggers *in vitro* and *in vivo*, and whether ALV infection blocks Type I IFN pathway.

Host pattern recognition receptors (e.g. toll-like receptors) recognize their pathogen cognates and initiates the production of interferons (IFNs), which bind to their receptors (IFNAR1/2), activating the JAK-STAT pathway, and transcriptionally induces hundreds of interferon-stimulated genes (ISGs) (O'Neill and Bowie, 2010; Qu et al., 2013). IFN treatment therefore induces a large set of ISGs that protect the host from infection with a broad range of viruses. ChIFNα inhibits the replication of many epidemic avian viruses *in vitro* and *in vivo*. These include avian influenza virus (AIV), infectious bursal disease virus (IBDV), infectious bronchitis virus (IBV), Marek's disease virus (MDV) and Newcastle Disease virus (NDV) (Jarosinski et al., 2001; Jiang et al., 2011; Meng et al., 2011; Mo et al., 2001; Pei et al., 2001). Nevertheless, until recently, the role of ChIFNα during ALV replication has not yet been examined.

In the present study, we determined *in vivo* cytokine mRNA expression levels in the late infection phase of ALV-J in chickens and ChIRF-3 promoter activation induced by ALV *in vitro*. To examine the antiviral effects of ChIFNα on ALV replication, we purified recombinant chicken interferon-α (rChIFNα) protein and measured its activity against vesicular stomatitis virus (VSV), Indiana serotype. We measured the *in vitro* inhibition of viral p27 protein production and RNA synthesis of ALV in DF-1 cells by rChIFNα. This is the first reported study of the effects of IFN-α on ALV infection.

2. Materials and methods

2.1. Ethics statement

The chicken sampling procedures were approved by South China Agriculture University's Institutional Animal Care and Use Committee.

2.2. Cells, viral stocks and reagents

Escherichia coli strain BL21 (DE3), pPET-23b (Novagen, EMD-Millipore) vector and VSV Indiana serotype were kept in our laboratory. DF-1 cells (American Type Culture Collection, Manassas, VA, USA) were grown in Dulbecco's modified Eagle medium (DMEM; GIBCO, Shanghai China) supplemented with 10% foetal bovine serum (FBS; GIBCO, Shanghai China) at 37 °C in a 5% CO_2 atmosphere. DF-1 is a chicken embryo fibroblast cell line free of endogenous sequences related to avian sarcoma and leukosis viruses. ALV-A strain GD13-1, ALV-B strain CD08, ALV-J strains CHN06 and NX0101 were propagated and quantified as described previously (Dai et al., 2016).

2.3. Cloacal swabs and tissue samples from ALV-J infected SPF chickens

To monitor virus shedding, cloacal swabs were collected from chickens from 2 weeks to 30 weeks, and preserved in the diluent of ALV-P27 Ag Test kit (IDEXX, Inc., Westbrook, MA). p27 expression levels in cloacal swabs were examined following the manufacturer's instructions. Tissue samples from 30-weeks-old specific-pathogen-free (SPF) chickens were prepared as described previously in the material and methods (Dai et al., 2015). Nine chickens infected with ALV-J strain CHN06 but without tumors were identified as the CHN06 group. Ten chickens without virus infection pertains to the control group. The tumor group contained 2 cases infected with ALV-J strain NX0101 and one with CHN06. Tissue samples from infected groups were evaluated by routine PCR virus culture isolation methods. The presence of p27 antigen and real-time PCR assays for viral sequences were all positive while control groups were all negative. In the CHN06 group, the viral gene load of bursa was higher than that of spleen. In the tumor case, both spleen and bursa had a medium viral load (Dai et al., 2015).

2.4. Cytokine measurement by qRT-PCR

The cytokine mRNA levels in chicken bursa and spleen tissues were quantified using qRT-PCR. The primers for the cytokine-specific amplification are shown in Table 1. The detailed steps of the SYBR Green I real-time PCR assay have been previously described (Dai et al., 2015).

2.5. Construction, expression and purification of recombinant rChIFN

The 489bp ChIFNα gene sequence retrieved from GenBank database (AB021154) was artificially synthesized and adjusted to the GC content and synonymous codon bias of *E. coli* by Huada Gene Research Institute (Shenzhen, China). The synthesized gene was then cloned into the *E. coli* expression vector pPET-23b (Novagen, EMD-Millipore) utilizing the *NdeI* and *EcoRI* sites which added a 6 × His tag at the 3′ end of the insert. The recombinant expression plasmid pET23b–rChIFNα was sequenced and had the predicted nucleotide sequence.

E. coli strain BL-21 (DE3) containing pET23b–rChIFNα was induced by isopropyl beta-D-thiogalactopyranoside (IPTG) at a final concentration of 1 mM at 37 °C. The induced BL-21 cells were harvested at 6 h by centrifugation at 8000 rpm for 10 min at 4 °C. The bacterial pellet was suspended in buffer A (50 mM Tris, 0.5 mM EDTA, 50 mM NaCl, pH 7) and lysed using an ultrasonic processor on ice for 30 min by alternating 3 s pulses with 3 s rests. The lysate was then centrifuged to collect precipitates. The resulting inclusion bodies were dissolved in buffer B (50 mM Tris-HCl, 8 M Urea, 5 mM DTT pH 8.0) and incubated for 12 h at 4 °C. Nickel–nitrilotriacetic acid (Ni–NTA) metal-affinity chromatography was used to purify 6 × His rChIFNα in accordance with the manufacturer's instructions (Qiagen). The eluted protein solution was dialyzed against four graduated changes of 1 L PBS containing 5, 3 and 1 M urea in sequence and lastly four times against 1 L PBS buffer. Finally, the sample was centrifuged and the supernatant containing rChIFN was filtered through a 0.22 μm filter, and then stored at—80 °C until use. Protein concentrations were determined by the BCA protein assay (Fermentas, Life Technologies).The purified recombinant ChIFNα protein was analyzed *via* SDS–PAGE using established protocols in Molecular Cloning (the 2nd edition). Western blotting was processed with monoclonal anti-His antibodies (Tiangen, Beijing, China) as the first antibody and IRDye 800-conjugated anti-mouse IgG (1:10,000; Rockland Immunochemicals, Limerick, PA, USA) diluted in PBS as the secondary antibody.

2.6. Bioactivity assay of recombinant rChIFNα

To determine whether the synthesized protein was biologically active, we used an antiviral assay based on the ability of recombinant ChIFNα to inhibit the VSV cytopathic effects on DF-1 cells.