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The pathogenic and vaccine strains of equine infectious anemia virus differentially induce cytokine and chemokine expression and apoptosis in macrophages

Yue-Zhi Lin, Xue-Zhi Cao, Liang Li, Li Li, Cheng-Gang Jiang, Xue-Feng Wang, Jian Ma, Jian-Hua Zhou*

State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin, China

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ABSTRACT

The attenuated equine infectious anemia virus (EIAV) vaccine was the first attenuated lentivirus vaccine to be used in a large-scale application and has been used to successfully control the spread of equine infectious anemia (EIA) in China. To better understand the potential role of cytokines in the pathogenesis of EIAV infection and resulting immune response, we used branched DNA technology to compare the mRNA expression levels of 12 cytokines and chemokines, including IL-1α, IL-1β, IL-4, IL-10, TNF-α, IFN-γ, IP-10, IL-8, MIP-1α, MIP-1β, MCP-1, and MCP-2, in equine monocyte-derived macrophages (eMDMs) infected with the $EIAV_{DLV121}$ vaccine strain or the parental $EIAV_{DLV34}$ pathogenic strain. Infection with $EIAV_{DLV34}$ and $EIAV_{DLV121}$ both caused changes in the mRNA levels of various cytokines and chemokines in eMDMs. In the early stage of infection with $EIAV_{DLV34}$ (0–24 h), the expression of the pro-inflammatory cytokines TNF-α and IL-1β were significantly up-regulated, while with $EIAV_{DLV121}$, expression of the anti-inflammatory cytokine IL-4 was markedly up-regulated. The effects on the expression of other cytokines and chemokines were similar between these two strains of virus. During the first 4 days after infection, the expression level of IL-4 in cells infected with the pathogenic strain were significantly higher than that in cells infected with the vaccine strain, but the expression of IL-1α and IL-1β induced by the vaccine strain was significantly higher than that observed with the pathogenic strain. In addition, after 4 days of infection with the pathogenic strain, the expression levels of 5 chemokines, but not IP-10, were markedly increased in eMDMs. In contrast, the vaccine strain did not up-regulate these chemokines to this level. Contrary to our expectation, induced apoptosis in eMDMs infected with the vaccine strain was significantly higher than that infected with the pathogenic strain 4 days and 6 days after infection. Together, these results contribute to a greater understanding of the pathogenesis of EIAV and of the mechanisms by which the immune response is induced after EIAV infection.

1. Introduction

Cytokines, including chemotactic, pro-inflammatory, and antiviral cytokines, are involved in the body's immune response to viral infections (Julkunen et al., 2001). As important immune regulatory factors, cytokines mediate the establishment of an antiviral state and recruit inflammatory cells to the site of infection (Alcami and Lira, 2010; Mackay, 2001; Yoshie et al., 2001). A number of studies have found that following infection with various viruses, such as human immunodeficiency virus-1 (HIV-1), simian immunodeficiency virus (SIV), equine viral arteritis (EVA), and feline immunodeficiency virus (FIV), the expression of a variety of cytokines and chemokines is imbalanced. Further studies confirmed that the abnormal expression of these cytokines and chemokines was closely related to disease progression (Qin et al., 2010; Quinlivan et al., 2007; Schmidtmayerova et al., 1996; Scott et al., 2010). For example, high expression of TNF-α and IL-10 were observed in HIV-1-infected individuals. The expression level of these cytokines positively correlated with the clinical disease status. Macrophage-derived cytokines and chemokines can also regulate the replication of viruses such as HIV-1 and SIV in monocytes and T lymphocytes (Peterson and Chesebro, 2006; Qin et al., 2010; Vicenzi et al., 1997), and therefore enhance the pathogenicity of viruses. These findings further demonstrated that cytokines and chemokines impact the outcome of viral infection.

Comparatively few studies have been published on equine infectious anemia virus (EIAV) infection and the resulting cytokine expression. EIAV can infect circulating monocytes, but effective replication of the virus is limited to differentiated tissue-derived

* Corresponding author at: State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, 427 Maduan Street, Harbin 150001, China. Fax: +86 0451 51997166.
E-mail address: jianhua_uc@126.com (J.-H. Zhou).

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macrophages. These macrophages are both the reservoir and the target population of the virus (Fidalgo-Carvalho et al., 2009; Sellon, 1993). Monocytes and macrophages are the main source of many cytokines. These cytokines can have an impact on the polarization and function of immune cells (Kopf et al., 2010; Yoshie et al., 2001). The expression levels and effects of pro- and anti-inflammatory cytokines are complexly balanced in physical status. The loss of this balance is closely related to disease pathology (Kedzierska and Crowe, 2001; Suresh and Wanchu, 2006; Yoshie et al., 2001). In fact, an in vitro study of EIAV identified significant changes in inflammatory cytokine responses in the early stages of infection (0.5–1 h) (Lim et al., 2005). Another study of EIAV confirmed that the thrombocytopenia associated with EIA infection in horses was not simply due to immune-mediated platelet destruction. Indeed, high expression of TNF-α, TNF-β, and IFN-α in the blood inhibits megakaryocytopoiesis (Tornquist et al., 1997). Although studies have compared the mRNA levels of IL-6, IL-1α, IL-1β, IL-10, and TNF-α in pathogenic and non-pathogenic EIAV strains after infection using RPA (ribonuclease protection assay) technology, several important pro-inflammatory cytokines, anti-inflammatory cytokines, and chemokines that have been associated with other lentiviral infections and pathogenicity were not included (Lim et al., 2003, 2005). The changes in the expression of cytokines and chemokines after the activation of mononuclear macrophages by EIAV infection and the impact on viral pathogenicity are still unclear. A further understanding and exploration of the relationship between cytokines and chemokines and viral infection-induced apoptosis, inflammation, and the overall immune response is one of the focuses in the field of EIAV research.

The attenuated EIAV vaccine is the first successful lentivirus vaccine. It was derived from a pathogenic strain isolated from the wild. After a series of in vivo and in vitro passages, a loss of virulence and the ability to induce protective immunity were achieved. This attenuated vaccine strain can effectively prevent the lethality of pathogenic strains (Ma et al., 2009, 2011; Shen et al., 2006). Therefore, this attenuated EIAV vaccine and pathogenic EIAV strains can be used as a unique model for researching immune protection against lentivirus. In this study, the expression of 12 cytokines and chemokines, including IL-1α, IL-1β, IL-4, IL-10, IFN-γ, TNF-α, IL-8, IP-10, MCP-1, MCP-2, MIP-1α, and MIP-1β was detected in equine monocyte-derived macrophages (eMDM) after in vitro infection with these two virus strains using the branched DNA (bDNA) technology developed by Panomics Inc. We aimed to explore two problems: (1) the impact of EIAV infection and replication on the expression of various cytokines and chemokines in target cells (monocytes/macrophages); and (2) differences between the attenuated vaccine and pathogenic strains in the impact on cytokines and chemokines expression. An understanding of these questions will provide important insight into the mechanisms of EIAV pathogenicity and the protective mechanisms of the attenuated vaccine.

2. Materials and methods

2.1. Cell culture

The primary culture of eMDMs was prepared as described by Lim et al. (2003). Briefly, equine peripheral blood from three healthy horses was collected and centrifuged at 1000 rpm for 10 min to separate the buffy coat. Equine peripheral blood mononuclear cells (PBMC) were isolated from the buffy coats by centrifugation through a HybriMax Histopaque cushion ($d = 1.077$ g/cm^3). Isolated PBMC were washed three times with PBS, resuspended in RPMI-1640 medium supplemented with 10% adult horse serum, 0.25 mM sodium HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate. Aliquots of PBMC were then seeded into tissue culture flasks at 5×10^6 cells/cm^2 and were incubated overnight at 37 °C, 5% CO2. Nonadherent cells were removed and adherent monocytes were further incubated for 3 days to allow differentiation into eMDMs. Cells were subsequently transferred to 48-well plates at 2×10^5 cells/well for cytokine and chemokine induction with EIAV.

2.2. Infection of eMDMs with EIAV

The amount of EIAV for infecting eMDM was tittered on fetal donkey dermal (FDD) cells using plaque-forming assay as previously described (Jiang et al., 2011). Three days post-plating, eMDMs were infected with 0.1 plaque-forming unit (PFU) of either $EIAV_{DLV34}$ or $EIAV_{DLV121}$. Cell culture medium was used to mock-infect eMDMs as a negative control. Cells were collected at 0 h, 2 h, 6 h, 24 h, and 2, 4, 6 days after infection and were lysed with Lysis Mixture (Affymetrix) for the detection of cytokine and chemokine expression. Cell culture media were collected at the same sampling points for the detection of viral replication levels. All samples were stored at −80 °C until required for analysis.

2.3. Replication of the pathogenic and vaccine strains of EIAV in eMDMs

The viral RNA copy numbers in the culture media were determined by quantitative real-time RT-PCR. The following oligonucleotide primers were used for the RT-PCR reactions: EIAV-gag-specific forward primer: 5′-CGA TGC CAA ATC CTC CAT TAG-3′, EIAV-gag-specific reverse primer: 5′-CTG ATC AAA AGC AGG TTC CAT CT-3′, and the TaqMan probe (supplied by BioSystems Inc., Paris, France) 5′-FAM-CAC CAC AAG GGC CTA TTC CCA TGA CA-TAMRA-3′. RT-PCR was performed using the one-step PrimeScript RT-PCR Kit (Takara, Shiga, Japan) in a 25 μl reaction mixture containing 12.5 μl of 2× RT-PCR buffer, 0.4 μM forward primer, 0.4 μM reverse primer, 0.2 μM TaqMan probe, 0.5 μl RT Mix *Ex-Taq* 0.5 μl, and 4 μl of the RNA sample or an RNA standard. The amplification protocol was carried out under the following conditions: 5 min at 42 °C, 10 s at 95 °C, and 40 cycles of 95 °C for 5 s and 60 °C for 40 s. Linear regression analysis of the standard curve was used to estimate the number of viral genomic RNA copies/ml of media. The standard RNA curve was linear in the range between 10^2 molecules at the lower limit and 10^9 molecules at the upper limit. All samples were measured in three independent experiments.

2.4. Measurement of cytokine and chemokine expression by bDNA assay

The bDNA assay is a sandwich nucleic acid hybridization platform in which target mRNA molecules are captured through cooperative hybridization of multiple probes. Published results have demonstrated that this assay is suitable to obtain reliable measurements of multiple-gene expressions simultaneously (Canales et al., 2006; Knudsen et al., 2008).

In this study, standard probe design software was used to design specific oligonucleotide probe sets for target genes for use in QuantiGene 2.0 Reagent Systems (Panomics, Inc., Fremont, CA) (see Table 1 for the sequences of probes). bDNA analysis was performed using the reagents provided by the manufacturer in a three-step procedure, which included specimen preparation, hybridization, and detection. Briefly, treated (stimulated with $EIAV_{DLV34}$ or $EIAV_{DLV121}$) or untreated eMDMs in 48-well plates were mixed with 80 μl lysis mixture and incubated at 60 °C for 1 h to release mRNA. Aliquots of 80 μl lysate were transferred to capture plates, which contained 20 μl pooled specific probes, and were incubated for hybridization with the probes for 20 h at 54 °C. The hybridization mixtures were removed, and microspheres in the