Veterinary Immunology and Immunopathology 180 (2016) 21–28

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

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

Marek's disease virus immunosuppression alters host cellular responses and immune gene expression in the skin of infected chickens

Mohammad Heidari [a,*], Dan Wang [b], Phillip Delekta [a], Shuhong Sun [b]

[a] United States Department of Agriculture, Agriculture Research Service, Avian Disease and Oncology Laboratory, East Lansing, MI, 48823, United States
[b] College of Animal Science and Veterinary Medicine, Shandong Agricultural University, Tai'an, Shandong, China

ARTICLE INFO

Article history:
Received 2 November 2015
Received in revised form 5 August 2016
Accepted 31 August 2016

Keywords:
Marek's disease
Immunosuppression
Feather follicle epithelium
Adhesion molecules
CD8 glycoprotein
Cytokines

ABSTRACT

Marek's disease virus (MDV), a highly cell-associated lymphotropic α-herpesvirus, is the causative agent of Marek's disease (MD) in domestic chickens. MDV replicates in chicken lymphocytes and establishes a latent infection within $CD4^+$ T cells. The latently infected $CD4^+$ T cells carry the virus to visceral organs, peripheral nerves, and feather follicle epithelium (FFE). FFE is the only anatomical site where infectious enveloped cell-free virus particles are produced and disseminated into the environment. This study investigated the immunological responses and mechanism of viral-induced immunosuppression and immune evasion in the FFE. Strong viral replication and lack of a significant number of cytotoxic T lymphocytes (CTL) in the infected tissues was prominent. Although the overall gene expression pattern was suggestive of a Th1 type immune response, the expression levels of several key immune genes were down regulated in the infected tissues. The mechanism of MDV-induced immunosuppression appears to be through inhibition of CTL function due to down regulation of CD8 glycoprotein and/or blocking of CTL migration due to decrease expression of cell adhesion molecules.

1. Introduction

Marek's disease (MD), one of the most common lymphoproliferative, ubiquitous, and neuropathic diseases of domestic chickens, is caused by a highly contagious cell-associated immunosuppressive α-herpesvirus, MD virus (MDV) or Gallid herpesvirus 2 (Churchill and Biggs, 1967; Nazerian et al., 1968). After a burst of productive/restrictive cytolytic infection in the B cells, a latent infection in $CD4^+$ T lymphocytes follows around 7 days post infection (dpi) that could last up to three weeks prior to virus reactivation and transformation phases of infection (Calnek, 2001; Ross, 1999). The lytic phase of the infection is characterized by the atrophy of lymphoid organs, inflammatory responses within the spleen, and a transient immunosuppression. The reactivation phase of MDV is exhibited as a second cycle of lytic infection, tumor development, and permanent immunosuppression (Schat and Nair, 2008; Schat et al., 1991). The latently infected T cells that harbor MDV migrate through the bloodstream and establish lymphomas in the skin, visceral organs, and peripheral nerves (Calnek, 2001). The feather follicle epithelium (FFE) is the only anatomical site where fully infectious enveloped cell-free virus particles are produced and released into the environment via stratified squamous epithelium that commonly slough off or detach with molted feathers (Calnek et al., 1970; Nazerian and Witter, 1970; Witter et al., 1972). A significant number of virus genome can be detected in the feather tips and FFE as early as 4 dpi (Abdul-Careem et al., 2008; Baigent et al., 2005) peaking between 3 and 4 weeks post infection (Baigent et al., 2013; Islam and Walkden-Brown, 2007; Witter et al., 1970). The expression of MDV antigens and the release of cell-free virus particles from skin can be detected throughout the life of the infected birds (Calnek et al., 1970).

Previous studies have shown that infection with very virulent oncogenic strains of MDV induces immunological responses within the feather tips characterized by up regulation of cytokines and infiltration of $CD4^+$ and $CD8^+$ T lymphocytes (Abdul-Careem et al., 2008). Although MD vaccination prevents lymphoma formation in addition to induction of a significant reduction in virus replication in the FFE, it does not induce sterile immunity and consequently, infectious virus particles remain in the host and are released into the environment to infect other birds (Baigent et al., 2013; Calnek

* Corresponding author at: USDA, Agricultural Research Service, Avian Disease and Oncology Laboratory, 4279 East Mount Hope Road, East Lansing, Michigan 48823, United States.
E-mail address: mohammad.heidari@ars.usda.gov (M. Heidari).

http://dx.doi.org/10.1016/j.vetimm.2016.08.013
0165-2427/

et al., 1970; Haq et al., 2012; Lee et al., 1999; Witter et al., 1971). Likewise, host responses induced in the skin of MDV-infected chickens are not effective enough to prevent viral replication in the FFE and dissemination into the environment (Baigent and Davison, 2004; Islam et al., 2006). The MDV-induced immunosuppression characterized by severe lymphopenia due to the massive destruction of B and T lymphocytes and down regulation of CD8 and MHC molecules undoubtedly has dire consequences for the biological functions of the effector cells of the immune system against MDV infection (Calnek et al., 1998; Morimura et al., 1995; Morimura et al., 1996).

The objective of the present study was to further investigate the immunological responses in the skin of MDV-infected chickens and provide insight into molecular mechanism of viral-induced immunosuppression and immune evasion. The generation of immune responses was assessed by gene expression profiling and immunohistochemical analysis of effector immune cell infiltration to the site of infection in the skin. The possible underlying mechanism of immune evasion and the lack of a vigorous effective cell-mediated immune response are discussed.

2. Material and methods

2.1. Experimental chickens

Chickens were F1 progeny ($15I_5X7_1$) of the Avian Disease and Oncology Laboratory (ADOL) lines $15I_5$ males and 7_1 females. The $15I_5X7_1$ birds were from unvaccinated breeder hens and carried no maternal antibodies to MDV or to herpesvirus of turkey. Chicks were hatched at ADOL poultry facility and housed in modified Horsfall-Bauer isolation units for the duration of the experiment.

2.2. Virus

A Bacterial Artificial Chromosome (BAC)-cloned very virulent (vv) strain of MDV, rMd5-BAC, which is propagated and maintained in ADOL laboratory, was used in this experiment (Niikura et al., 2011).

2.3. Immunohistochemistry

Samples previously flash frozen in embedding medium, Optimal Cutting Temperature (OCT) (Sakura Finetek, Torrance, CA), were sectioned on a cryotome at 5 μm and placed on slides coated with 2% 3-Aminopropyltriethoxysilane and air dried at 25 °C overnight. Subsequently microtome sections were fixed in formal acetate fixative for 10 min at room temperature followed by 3 changes of Tris buffered saline – 5 min each. Endogenous peroxidase activity was blocked with 0.3% Hydrogen peroxide in Tris buffered saline for 20 min followed by tap and distilled water rinses. Following pretreatment standard, Avidin-Biotin complex staining steps were performed at room temperature on the DAKO Autostainer (Agilent Technologies, Carpentaria, CA). All staining steps were followed by rinses in Tris buffered saline + tween 20 (Scytek Laboratories, West Logan, UT). After blocking for non-specific protein with normal horse serum (1/30 dilution in PBS; Vector Labs, Burlingame, CA) for 30 min, sections were incubated with Avidin/Biotin blocking system for 15 min each (Vector Lab, Burlingame, CA; Sigma, St. Louis, MO). Samples were then incubated with mouse anti-chicken B cell, $CD4^+$ T cell, $CD8^+$ T cells (CD8α and CD8β), γd T cell, or macrophage primary monoclonal antibodies (Bu-1/chB6, CT-4, CT-8, EP42, TR-1, and KUL01, respectively; SouthernBiotech, Birmingham, AL) for 1 h in Normal Antibody Diluent (NAD) (Scytek Laboratories, West Log, UT) followed by rinsing and incubation with biotinylated horse anti-mouse IgG (H + L) prepared at 11.5 μg/ml in NAD incubated for 60 min. For detection of MDV antigen, mouse anti ribonucleotide reductase (RR) monoclonal antibody (T81) (Lee et al., 1983) was used. Samples then were incubated with R.T.U. Vector Elite Peroxidase Reagent (Vector Laboratories, Burlingame, CA) for 30 min. Reaction development utilized Vector Nova Red peroxidase chromogen incubation of 15 min followed by counterstain in Gill Hematoxylin (Thermo Fisher – Kalamazoo, MI) for 15 s, differentiation, and dehydration, clearing and mounting with synthetic mounting medium. The working dilution for all monoclonal antibodies specific for chicken cells was 1:200. The working dilution for the monoclonal antibody specific for MDV RR was 1:2000.

2.4. RNA isolation

Total RNA was isolated from the homogenized skin tissues (breast skin with secondary flight feathers) of three birds of each group (See experimental design) at 26 days post infection (three biological replications) using Tri Reagent RT (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. The feathers from collected skin samples were clipped at the base with tips remaining in the skin.

2.5. Real-time RT-PCR

Real-time PCR analysis of relative quantification of gene expression was performed in transcripts for each biological sample at the Research Technology and Support Facility of Michigan State University in East Lansing, Michigan. Briefly, 2.5 μL of a 1:20 dilution of the oligo dT-based RT product from 4 μg of total RNA isolated from skin tissues was used for each reaction. 300 nM of each specific sense and anti-sense primers were used in the presence of 5 μL SYBR Green PCR master mix (Applied Biosystems, Foster City, CA). The amplification program was as follows: 50 °C for 2 min, 95 °C for 10 min, 40 cycles at 95 °C for 15 s, followed by 57 °C for 1 min. All the reactions were run in triplicates in a 7900HT Sequence Detection System (Thermo Fisher Scientific, Grans Island, NY). The primers for chicken genes were designed using MacVector software (Accelrys, San Diego, CA). All the primers were synthesized by Operon Biotechnologies, Inc. (Huntsville, AL). The primer sequences are listed in Table 1. Relative quantification of the chicken genes was determined using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The levels of gene expression in the skin tissues of age-matched control birds were used as reference or baseline for calculation of fold changes in gene expression in MDV-inoculated chickens. The expression of each gene was normalized to the expression level of the housekeeping gene, β-actin. The calculation for fold change in the expression of each gene was based on the average CT values from three samples (Three biological replications) and three replicates for each sample (Three experimental replications).

2.6. Experimental design

One-day-old chicks were randomly distributed into 2 groups of 10 each in separate isolators. Birds from one group were inoculated intraperitoneally with 2000 pfu of rMd5 at 12 days post hatch. The second group served as un-inoculated negative control. Day-old chicks were not used because the immune system is not fully developed or activated at hatch. At 26 dpi, three birds from each group were euthanized by CO_2 inhalation and necropsied for tissue collection. Due to the high mortality rate among the MDV-infected chickens, we started with more birds per group than the actual numbers used for sample collection. A portion of each sample was stored in RNAlater (Thermo Fisher Scientific, Grans Island, NY) to prevent RNA degradation. A small section of each skin sample was also embedded in OCT embedding medium (Sakura Finetek USA, Inc. Torrance, CA) and snap-frozen in liquid nitrogen and stored in −80 °C until used for immunohistochemical analysis. MDV in the