Veterinary Immunology and Immunopathology 136 (2010) 305–310

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

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

Short communication

Increased numbers of myeloid and lymphoid IL-10 producing cells in spleen of pigs with naturally occurring postweaning multisystemic wasting syndrome

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ARTICLE INFO

Article history:
Received 7 August 2009
Received in revised form 2 March 2010
Accepted 8 March 2010

Keywords:
Postweaning multisystemic wasting syndrome
Porcine circovirus type 2
Immunofluorescence assay
Interleukin-10
Spleen

ABSTRACT

Porcine circovirus type 2 (PCV2) is the essential etiological agent of postweaning multisystemic wasting syndrome (PMWS), a worldwide distributed pig disease. The involvement of the immune system in the pathogenesis of PMWS is considered crucial. Previous studies have shown a cytokine profile suggesting T immunosuppression and indicating that interleukin 10 (IL-10) may play an important role during PCV2 infection. Nine 11- to 12-week-old conventional pigs were obtained from commercial farms located in North-Eastern Spain with historical records of PMWS. Spleen from four healthy and five PMWS-affected animals were collected at the necropsy. Viral load was determined in serum by means of standard PCR and real-time quantitative PCR. Phenotype and distribution of different immune cells involved in IL-10 secretion in the spleen of studied pigs were analysed using immunofluorescent assays. The $CD163^+$, $CD4^+$, and $CD8^+$ cell subpopulations produced IL-10 in the spleen and $IL\text{-}10^+$ cell numbers were higher in PMWS animals compared with their healthy counterparts. Furthermore, IL-10 producing cells were not infected by PCV2 and were mainly localized in the periarteriolar lymphoid sheaths. This is the first immunophenotyping study on IL-10 producing cells in cases of PMWS, further extending the studies on the role of IL-10 in disease pathogenesis.

1. Introduction

Postweaning multisystemic wasting syndrome (PMWS) is a worldwide distributed disease of pigs with major economic impact in swine production (Segalés et al., 2005). The essential etiological agent of this condition is porcine circovirus type 2 (PCV2), a single stranded DNA virus belonging to the *Circoviridae* family. PMWS is, however, defined as a multifactorial condition, since the experimental reproduction of the disease and even its occurrence under field conditions usually require a number of risk or triggering factors besides the viral infection (Tomas et al., 2008).

The most characteristic pathological features of PMWS are lymphocyte depletion and granulomatous inflammation of lymphoid organs (Rosell et al., 1999). Recent studies have suggested that lymphocyte-like cells may be an important cell populations that support early PCV2 replication, whereas monocytes may be the site for PCV2 persistence in the infected host (Yu et al., 2007).

The extensive lymphoid lesions (Rosell et al., 1999) and the association of PMWS with secondary and opportunistic infections (Carrasco et al., 2000) support the involvement of the immune system in the pathogenesis of PMWS. However, only few studies have described parameters of

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doi:10.1016/j.vetimm.2010.03.008

Table 1
Primary antibodies (immune subpopulation phenotyping) used in the immunofluorescence assays. HSN, hybridoma supernatant; PUR, purified; PAb, polyclonal antibody.

Primary antibodies	Specificity	Source	Dilution	Reference
CD3 (PAb)	T lymphocytes	PUR Dako (Denmark)	1:50	Chianini et al. (2003)
CD4 (74-12-4) IgG2b	Naïve, memory/activated Th	HSN INIA (Spain)	1:5	Pescovitz et al. (1984)
CD8α (76-2-11) IgG2a	Memory Th, Tc, NK cells	HSN INIA (Spain)	Neat	Pescovitz et al. (1984)
CD163 (2A10/11) IgG1	Monocytes and macrophages	HSN INIA (Spain)	Neat	Bullido et al. (1997)

cell-mediated immunity in clinical cases of PMWS, supporting the idea of immune system impairment (Darwich et al., 2003). Nevertheless, there are many *in vitro* evidences of the immunomodulatory capacity of PCV2. This virus has the ability to suppress the *in vitro* release of some cytokines in both healthy and diseased pigs, and it is able to stimulate the production of pro-inflammatory cytokines (Darwich et al., 2003). Peripheral blood mononuclear cells (PBMCs) from PMWS-affected pigs are less able to produce IL-2, IL-4 and IFN-γ upon mitogen or superantigen challenges and they are able to produce IL-10 after stimulation with recall viral antigens. This fact and the inability to produce IL-2 and IL-4 could be indications of the activity of regulatory or suppressing cells (Darwich et al., 2003). Additionally, different components of PCV2 have been shown to play an important role in the modulation of the *in vitro* responses by PBMCs (Kekarainen et al., 2008a).

A recent *in vitro* experiment investigating the modulation of immune system of PCV2 during recall responses against pseudorabies virus (PRV) confirmed that PCV2-induced IL-10 may participate in down-regulation of specific responses through the inhibition of IFN-γ, IFN-α and IL-12. In addition, PCV2 is able to inhibit IL-2 through an IL-10 independent mechanism (Kekarainen et al., 2008b). *In vivo* data indicate that PMWS-affected animals also show elevated serum IL-10 levels (Hasslung et al., 2005) and subclinically PCV2-infected pigs develop a transient IL-10 PCV2-specific response during the viremic phase of the infection (Darwich et al., 2008).

Considering the IL-10 relevance showed recently, the main objective of this work was to study and to characterize the distribution of different immune cell subpopulations involved in IL-10 secretion in the spleen of naturally occurring PMWS-affected pigs.

2. Materials and methods

2.1. Animals and tissue samples

Nine 11- to 12-week-old conventional pigs were obtained from commercial farms with historical records of PMWS. Herds were located in North-Eastern Spain and all the animals, at the moment of the sampling, were seronegative for Aujeszky disease virus (ADV), negative for porcine reproductive and respiratory syndrome (PRRSV) and swine influenza viruses (SIV) by immunohistochemistry.

Five of the pigs were selected as confirmed cases of the disease, fulfilling the internationally accepted case definition criteria based on (i) clinical signs, (ii) presence of characteristic histopathological lesions in lymphoid tissues, and (iii) detection of moderate to high amounts of PCV2 within the lymphoid lesions (Segalés and Domingo, 2002). The other four pigs were used as controls and they were selected for their healthy condition, confirmed to be negative to PCV2 by *in situ* hybridization (Rosell et al., 1999) and with no PMWS-like lesions at histopathological examination. Spleen from those animals was collected at the necropsy and sections of about 0.5 cm^3 were placed into plastic moulds (Electron Microscopy Science, Hatfield, PA) surrounded in optimal cutting compound (Tissue-Tek O.C.T. Compound, Sakura Finetek Europe B.V., NL) before freezing in dry ice. Frozen samples were stored at −80 °C until used.

2.2. Antibodies and immunofluorescence assay

Indirect immunofluorescence assays were performed following standard procedures (Hofman, 2002). Briefly, frozen samples were cut at approximately 8–10 μm, air-dried and fixed with 2% paraformaldehyde. Sections were then permeabilized in 0.5% Triton X-100 and incubated in blocking solution. The immunophenotyping of IL-10 producing cells was performed using the mouse biotin-conjugated anti-swine IL-10 (BioSource, Camarillo, CA). On the other hand, the phenotype of PCV2-infected cells was determined using the mouse anti-PCV2 VP2 antibody (Ingenasa, Madrid, Spain). Different cell marker antibodies were concomitantly used with anti-IL-10 and/or anti-PCV2 antibodies (Table 1). CD3 cell marker antibody was detected with a goat anti-rabbit biotinylated antibody (Dako, Denmark) followed by TRITC-conjugated streptavidin (ZYMED® Laboratories, California). Anti-IL-10 biotinylated antibody was detected using the TRITC-conjugated streptavidin. The rest of primary antibodies against cell markers and PCV2 were detected using goat anti-mouse secondary antibodies coupled to FITC, Cy2 or Cy3 fluorophores (Jackson ImmunoResearch Europe Ltd.). Finally, nuclei were counterstained with DAPI.

Negative control procedures were applied in each tissue and for all immunofluorescent tests. Specifically, false positivity, autofluorescence, and cross-reactivity were assessed by means of (1) lack of all reagents; (2) lack of primary antibody; (3) negative isotype controls (IgG1, IgG2a, IgG2b) as irrelevant primary antibodies, and (4) swapping secondary antibodies for the primary combinations.

2.3. Microscopy, image and statistical analysis

Treated sections were viewed on a Nikon eclipse 90i epifluorescence microscope equipped with a DXM 1200F camera (Nikon Corporation, Japan). To assess the co-localization between different fluorescent labels, image stacks of sections were captured using a Leica TCS SP5 confocal microscope (40×/NA 1.25 objective). Z stack images