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Research paper

Immunohistochemical expression of IL-12, IL-10, IFN-α and IFN-γ in lymphoid organs of porcine reproductive and respiratory syndrome virus-infected pigs

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ABSTRACT

Despite the numerous studies carried out, the mechanisms used by porcine reproductive and respiratory syndrome (PRRS) virus (PRRSV) to impair the host immune response are not yet clear. The aim of this study was to determine the expression of IL-12, IL-10, IFN-α and IFN-γ in lymphoid organs of PRRSV experimentally-infected pigs. Twenty eight piglets were inoculated with PRRSV field isolate 2982 and killed in batches of four at 3, 7, 10, 14, 17, 21 and 24 days post-inoculation (dpi). Control animals were mock-inoculated and killed at the end of the study. Samples from mediastinal and retropharyngeal lymph nodes and tonsil were collected and fixed for histopathological and immunohistochemical analyses. PRRSV antigen was mainly detected in the cytoplasm of macrophages, displaying a bimodal expression with a first peak at 3–7 dpi and a second peak at 14 dpi. The expression of IFN-α showed an early enhancement at 3 dpi, and both IL-12 and IFN-γ displayed a similar trend in all the lymphoid organs analysed, showing an increase at 3–7 dpi and at 14–17 dpi. On the other hand, the expression of IL-10 was lower than the one observed for the other cytokines. The expression of IL-10 compared with the higher expression of IL-12, IFN-α and IFN-γ detected in this study, indicates that other mechanisms besides the expression of IL-10 play a role in the inducement of an erratic host immune response. Taking into account the enhanced expression of IFNs together with the detection of PRRSV antigen until the end of the study in the examined lymphoid organs, further studies are being conducted to rule out a down-regulation in IFN signalling pathway.

1. Introduction

Porcine Reproductive and Respiratory Sydrome (PRRS) which is caused by PRRS virus (PRRSV) (Fauquet et al., 2005), is one of the most economically significant diseases of the swine industry (Neumann et al., 2005). PRRSV induces an impairment of the host immune response

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favouring a prolonged viraemia and viral replication (Darwich et al., 2010); however, the exact mechanism involved in the modulation of the immune response still remains unclear. PRRSV replication has been reported in both lung and lymphoid organs of PRRSV-infected pigs (Xiao et al., 2004; Gómez-Laguna et al., 2010; Barranco et al., 2012), which suggests a role of these organs in the pathogenesis of the disease.

The production of cytokines is one of the main tools used by macrophages and also by several other immune or non-immune cells in the defense against pathogens (Kumar et al., 2008). IFN-γ and IL-12 are classically involved in the subtype of immune response mediated by Th1 lymphocytes, working both cytokines in parallel (Biron and Sen, 2001). As do proinflammatory cytokines, IFN-α participates in the innate response, triggering an antiviral activity by means of the differentiation of naïve T cells into IFN-γ secreting cells and the down-regulation of IL-12 expression (Biron and Sen, 2001; Tizard, 2008). In contrast, IL-10 is considered an immunosuppressive cytokine which down-regulates the expression of several other cytokines including IL-1α, TNF-α, IL-6, IL-10 itself, IL-12 and IFN-γ (Biron and Sen, 2001; Moore et al., 2001). Several studies have examined the role of cytokines in the pathogenesis of PRRS (Albina et al., 1994; Bautista and Molitor, 1999; Van Reeth and Nauwynck, 2000; Díaz et al., 2006); however, it is not clear how cytokines regulate the onset of the immune response against PRRSV. Albina et al. (1994) suggested that a downregulation of IFN-α production plays an important role in enabling PRRSV replication. Likewise, Bautista and Molitor (1999) reported a significant role of IFN-γ in the protection of host cells against viral replication. In this sense, an upregulation of the expression of IL-10 has been associated with a lower number of IFN-γ secreting cells amongst peripheral blood mononuclear cells (PBMCs) and a lower protection rate after challenging in pigs vaccinated with a modified-live vaccine (Díaz et al., 2006). However, most of the studies focused on the expression of cytokines after PRRSV infection have been performed in serum and lung samples (Batista et al., 2004; Xibao et al., 2010; Gómez-Laguna et al., 2010a) and in a lesser extent in the lymphoid tissues *in situ* (Rossow et al., 1996; Beyer et al., 2000).

Recently, our group has reported a lack of homogeneity in the proinflammatory cytokines response in lymphoid organs of PRRSV-infected pigs, which may be associated with the erratic immune response observed in the disease (Barranco et al., 2012). In the present study, samples from this previous animal experiment were used to determine the *in situ* expression of IL-12, IL-10, IFN-α and IFN-γ cytokines in the lymphoid organs of PRRSV-infected pigs and to determine their correlation with the expression of PRRSV antigen, in order to gain further insight in the immunopathogenesis of PRRS.

2. Materials and methods

2.1. Virus, animals and experimental design

Thirty-two specific pathogen free, 5-week-old pigs from a PRRSV seronegative farm were used in this study. Twenty-eight of them were randomly allocated in batches of four ($n = 4$), inoculated by intramuscular route with 1 ml of $10^{3.0}$ $TCID_{50}$ of the PRRSV field isolate 2982 (kindly provided by Dr. E. Mateu) and sequentially killed at 3, 7, 10, 14, 17, 21 and 24 days post-inoculation (dpi). Another four pigs, used as control animals, were injected intramuscularly with 1 ml of sterile RPMI (Roswell Park Memorial Institute medium) 1640 medium and killed at the end of the study (24 dpi). All animals were sedated with tiletamine-zolazepam (Zoletil™; Virbac, Barcelona, Spain) followed by intravenous injection of a lethal dose of 5% sodium thiopental (Thiovet™; Vet Limited, Leyland, Lancashire). This experiment was carried out according to the guidelines of the European Union (Directive 86/609/EEC) and was approved by the Local Ethics Review Committee of Centro de Investigación en Sanidad Animal (CISA-INIA; Valdeolmos, Madrid, Spain).

2.2. Clinical signs, gross pathology and histopathology

The pigs were monitored daily for clinical signs as previously described by Halbur et al. (1995). At the post mortem examination, macroscopic lesions were evaluated following the scoring system described by Halbur et al. (1995). Samples from tonsils, retropharyngeal lymph node and mediastinal lymph node were collected and fixed in 10% neutral buffered formalin and in Bouin's solution for the histopathological and immunohistochemical studies, respectively. Fixed samples were routinely processed and embedded in paraffin-wax.

2.3. Immunohistochemical study

The avidin–biotin-peroxidase complex technique (ABC) was used for the detection of PRRSV and cytokines as described previously (Gómez-Laguna et al., 2010). Briefly, 4 μm sections were dewaxed and rehydrated through graded ethanol solutions. The primary antibodies used were monoclonal anti-PRRSV (clone SDOW-17/SR-30; Rural Technologies Inc.) diluted 1 in 1000; polyclonal anti-pig IL-12 p-40 (RnD Systems) diluted 1 in 20; polyclonal anti-pig IL-10 (RnD Systems) diluted 1 in 20; monoclonal anti-pig IFN-α (clone F17; kindly provided by Prof. K. Van Reeth) diluted 1 in 300, and polyclonal anti-pig IFN-γ (RnD Systems) diluted 1 in 20. The antigen retrieval method used for all the cytokines studied was permeabilisation with Tween 20 diluted 0.01% in PBS during 10 min, but for monoclonal anti-PRRSV antibody a High Temperature Antigen Retrieval with citrate buffer ph 6.0 was used. Primary antibodies were incubated overnight at 4 °C in a humid chamber. In each case, the corresponding biotinylated secondary antibody was incubated for 30 min at room temperature. An avidin-peroxidase complex (Vector Laboratories, Burlingame, CA) was applied for 1 h at room temperature. Labelling was "visualized" by application of the NovaRED™ substrate kit (Vector Laboratories). Sections were counterstained with Mayer's haematoxylin, dehydrated and mounted. Primary antibody-omitted negative controls (replacing the primary antibody by blocking