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Interaction of PRRS virus with bone marrow monocyte subsets

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ABSTRACT

PRRSV can replicate for months in lymphoid organs leading to persistent host infections. Porcine bone marrow comprises two major monocyte subsets, one of which expresses CD163 and CD169, two receptors involved in the entry of PRRSV in macrophages. In this study, we investigate the permissiveness of these subsets to PRRSV infection. PRRSV replicates efficiently in BM CD163$^+$ monocytes reaching titers similar to those obtained in alveolar macrophages, but with a delayed kinetics. Infection of BM CD163$^-$ monocytes was variable and yielded lower titers. This may be related with the capacity of BM CD163$^-$ monocytes to differentiate into CD163$^+$ CD169$^+$ cells after culture in presence of M-CSF. Both subsets secreted IL-8 in response to virus but CD163$^+$ cells tended to produce higher amounts. The infection of BM monocytes by PRRSV may contribute to persistence of the virus in this compartment and to hematological disorders found in infected animals such as the reduction in the number of peripheral blood monocytes.

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is the etiological agent of an economically important infectious disease that affect the pig industry worldwide. It is characterized by severe reproductive disorders in sows and respiratory distress in young growing and finishing pigs, predisposing them to secondary infections associated with the porcine respiratory disease complex (Chand et al., 2012).

PRRSV is an enveloped positive-sense single-stranded RNA virus, which belongs to the Arteriviridae family in the order Nidovirales, and comprises two well defined genotypes: type 1 or European and type 2 or North American (Meng et al., 1995). Both genotypes cause similar clinical manifestations of reproductive failure and respiratory problems in pigs but share only about 60% of their identity of the nucleotide sequence and show differences in antigenicity. Recently, PRRSV type 1 and type 2 have been classified into two species within the genus Porartevirus: PRRSV-1 and PRRSV-2, respectively (Adams et al., 2016).

PRRSV shows a strong tropism for cells of monocyte-macrophage lineage (Duan et al., 1997b). The virus infects macrophages in lungs and several lymphoid organs, while freshly isolated blood monocytes are largely refractory. The different permissiveness to infection of these cells has been related to differences in the expression of receptors for the virus (Delputte et al., 2007; Duan et al., 1997a; Patton et al., 2009). CD169 (also named Siglec-1 or sialoadhesin) and CD163 have been identified as the most important host receptors for the entry and replication of PRRSV into alveolar macrophages (Van Gorp et al., 2008), the latter being essential for infection (Wells et al., 2017). Blood monocytes express variable amounts of CD163 and are usually negative for CD169, however the expression of these receptors can be induced after different treatments (Delputte et al., 2007; Ezquerra et al., 2009; Patton et al., 2009).

Most studies on the interaction of PRRSV with bone marrow (BM) cells have been carried out on cells that have been differentiated *in vitro* into macrophages or DCs, under diverse culture conditions (Chang et al., 2008; Chaudhuri et al., 2016; Gimeno et al., 2011). However, data on the interaction of the virus with undifferentiated BM cells are scarce. Early studies have reported the existence of lesions in the BM of PRRSV-infected piglets (Feng et al., 2001). More recently, viral RNA has been detected in BM of animals infected with either type 1 or type 2 isolates, suggesting viral replication in BM cells (Amarilla et al., 2017; Wang et al., 2016). Whereas viral antigens were occasionally detected in BM tissue sections from animals infected with PRRSV type 1 isolates (Amarilla et al., 2017), Wang et al found a marked expression of PRRSV N protein in CD172a$^+$ SWC8$^-$ BM cells of animals infected with the highly pathogenic-PRRSV type 2 HuN4 strain (Wang et al., 2016).

We have recently characterized two subsets of BM monocytes, one of which expresses both CD163 and CD169. The aim of this study was to investigate the permissiveness of bone marrow monocytes to infection

Abbreviations: BM, bone marrow; rhM-CSF, recombinant human macrophage colony stimulating factor

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by PRRSV type 1 isolate, and the effect of virus on cytokine production, as these cells may be an important target for PRRSV persistence *in vivo*.

2. Materials and methods

2.1. Animals, tissues and cells

Tissue samples were obtained from healthy, conventionally reared, 3 to 7-week-old Large-White pigs that had been anesthetized with sodium pentobarbital (0.3 g/Kg) by intraperitoneal route and euthanized by exsanguination. Alveolar macrophages were collected by bronchoalveolar lavage. BM cells were isolated from femurs, tibias and humeri by flushing with PBS containing 2 mM EDTA, 2% FCS and 150 μg/ml gentamicin, using a 20 ml syringe with an 18-g needle. These cells were washed twice and resuspended in 150 mM NH_4Cl, 10 mM $NaHCO_3$, pH 7.3 for 5 min with gentle shaking to lyse red blood cells. Afterwards, mature granulocytes were removed by centrifugation over a discontinuous 53% Percoll gradient. Remaining cells were then resuspended in RPMI 1640 medium containing 10% FCS, 2 mM L-glutamine, 20 mM Hepes, 5×10^{-5} M 2-mercaptoethanol and 50 μg/ml gentamicin (complete medium).

The reported experiments have been executed in full compliance with guidelines by the ethical and animal welfare committees of the Institute.

2.2. Antibodies and flow cytometry

Hybridoma producing monoclonal antibody (mAb) to CD172a (74-22-15a, IgG2b) was kindly provided by J. Lunney (ARS USDA, USA). MAbs to CD163 (2A10/11, IgG1), CD169 (1F1, IgG2a), CD172a (BA1C11, IgG1), and SLA-DR (1D2CR4, IgG2a) were produced in our laboratory. MAb to PRRSV N protein (1CH5, IgG2b) was purchased from Ingenasa (Madrid, Spain) and labelled with Alexa Fluor 488 dye following the manufacturer's protocol (Molecular Probes, Eugene, OR, USA). Irrelevant mAbs were used as negative controls.

For two-colour staining, cells were incubated with one mAb to CD163 (IgG1) and other to SLA-DR (IgG2a), CD169 (IgG2a) or CD172a (IgG2b) for 20 min at 4 °C. After two washes with PBS containing 0.1% bovine serum albumin and 0.01% sodium azide (fluorescence buffer), cells were incubated with APC-conjugated goat anti-mouse IgG1 and PE-conjugated goat anti-mouse IgG2a or IgG2b antibodies respectively (Southern Biotech, Birmingham, AL, USA) for 20 min at 4 °C. Afterwards, cells were washed twice and Sytox blue cell stain (Life Technologies) added prior to their analysis in a FASCanto II flow cytometer (BD Biosciences, San Jose, CA) to exclude dead cells.

For detection of the viral nucleoprotein, cells were permeabilized with methanol for 10 min at −20 °C and washed with fluorescence buffer. Then, they were incubated with 1CH5 mAb labelled with Alexa 488 at 4 °C. Finally, cells were washed and analysed in the cytometer. Non-specific binding was evaluated using an irrelevant mAb labelled with Alexa 488.

Data were analyzed using FlowJo software. Doublets were excluded using FSC-A *versus* FSC-H plots.

2.3. Sorting of bone marrow monocyte subsets

BM monocyte subsets were sorted as described before (Fernandez-Caballero et al., 2018). BM cells were incubated with mAbs anti-CD172a (74-22-15a) and anti-CD163 (2A10/11) for 20 min at 4 °C. After washing, cells were incubated with PE-conjugated goat anti-mouse IgG2b and APC-conjugated goat anti-mouse IgG1 (Southern Biotech, USA), for 20 min at 4 °C. After a final wash, cells were filtered through a 70 μm nylon strainer (Thermofisher Scientific, USA) for removal of cell clumps, prior to sorting. Dead cells were excluded with Sytox blue dead cell stain (Life Technologies, USA) and non-specific binding was evaluated using irrelevant control mAbs. Granulocytes were excluded based on characteristic FSC and SSC profiles. SSC^{lo} $CD172a^{hi}$ $CD163^-$ and $CD163^+$ cells were sorted simultaneously using a FACSAria III cell sorter (BD Biosciences, San Jose, CA) and collected in complete medium containing 20% FCS and 2 mM EDTA. Purity of the separated populations was higher than 95%.

2.4. In vitro maturation of monocytes

$CD163^-$ and $CD163^+$ sorted monocytes were cultured for 3 days in complete medium supplemented with 20 ng/ml of recombinant human (rh) M-CSF (Gibco Life Technologies, USA). At different times cells were harvested and analysed by flow cytometry with mAbs to CD163, CD169, CD172a or SLA DR.

2.5. PRRS virus infection and titration by qRT-PCR

PRRS virus type 1 isolate 5710, kindly provided by Dr. J.M. Castro, (Facultad de Veterinaria, UCM, Madrid, Spain), was used for all infections. Viral stocks were grown and titrated on porcine alveolar macrophages as previously reported (Yuste et al., 2017). Sorted monocytes or alveolar macrophages were infected with PRRSV at a multiplicity of infection (m.o.i.) of 0.5. After 1 h of incubation at 37 °C, cells were washed twice to remove non-attached virus and cultured in 48-well plates (5×10^5 cells/well in 500 μl of complete medium). At different time-points post infection (p.i.), cells and supernatants were harvested for analysis of infection. After three cycles of freezing and thawing, cell debris was removed by centrifugation at 3000 *g* for 5 min at 4 °C and virus samples were frozen and stored at −80 °C. Samples obtained at the time of plating, after 1 h of viral incubation, were considered time-zero samples. Virus content was determined by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). A QiAamp Viral RNA MiniKit (Qiagen, Manchester,UK) was used to extract RNA from 140 μl of virus samples. RNA was eluted into 60 μl of elution buffer and stored at −80 °C prior to RT-PCR analysis. PRRSV detection by qRT-PCR was performed using VetMax NA &EU PRRSV reagents for PRRSV N gene amplification (Thermo Fisher Scientific), adding 7 μl of RNA to 18 μl of the PCR master mix (containing specific forward/reverse primers and probe). qRT-PCRs were carried out using a 7500 Fast Real-time PCR System (Applied Biosystems, Thermo Fisher Scientific). PRRSV titers were determined by comparison of the obtained threshold cycle (CT) values to a standard curve generated by using 1/10 serial dilutions of a known amount of PRRSV and expressed as $TCID_{50}$/ml.

2.6. Cytokine production

Sorted monocytes were plated at 5×10^5 cells/well in 0.5 ml of culture medium in 48-well plates, and incubated with PRRSV at an m.o.i. of 0.5, or stimulated with poly I:C (10 μg/ml) or LPS (1 μg/ml, E. Coli O55:B5), (both from Sigma-Aldrich, St Louis, MO, USA). Cells incubated with supernatants from mock-infected PAM were used as negative controls. After 21 or 46 h, supernatants were collected, centrifuged to remove cell debris, and frozen at −80 °C until assessment of cytokine production. Supernatants were analyzed for IL-8, IL-10 and TNF-α cytokine content by using commercial ELISA kits from Invitrogen following manufacturer's instructions. ELISA for IFN-α was performed using monoclonal antibodies K9 and F17 from PBL Interferon Source, as previously described (Borrego et al., 2015). The amount of cytokines produced in response to virus was calculated by subtracting cytokine levels in mock-stimulated cultures.

2.7. Statistical analysis

Statistical analyses of data were performed with the Mann-Whitney rank test, using GraphPad Prism 4 software (La Jolla, CA, USA). All experiments were performed independently at least three times using cells isolated from different pigs, unless stated otherwise. Statistical