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Monocyte derived macrophages as an appropriate model for porcine cytomegalovirus immunobiology studies

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ABSTRACT

Porcine cytomegalovirus (PCMV) causes lifelong latent infections in swine. The pathogen is occasionally associated with inclusion body rhinitis and pneumonia in piglets, reproductive disorders in pregnant sows and respiratory disease complex in older pigs. Immunosuppressive potential of PCMV infection is discussed. Macrophages were recognised as one of target cell types where propagation of virus occurs. The aim of present study was to set up model PCMV infection of monocyte derived macrophages (MDMs) *in vitro* for PCMV immunobiology research. Obtained results showed that PCMV is able to infect and propagate in MDMs. Possible immunosuppressive effect of PCMV on infected macrophages was evaluated by measurement of immune relevant gene expression in MDMs. Infection decreased expression of IL-8 and TNF-α (pro-inflammatory cytokines) and increased expression of IL-10 (anti-inflammatory cytokine) on mRNA transcription level. Obtained data support hypothesis that higher sensitivity of animals to coinfection with other swine pathogens and its more severe clinical manifestations could potentially be the consequence of PCMV infection.

1. Introduction

Porcine cytomegalovirus (PCMV) causes lifelong latent infections in swine worldwide. Disease is usually mild and difficult to recognize, nevertheless pathogen is occasionally associated with inclusion body rhinitis and pneumonia in piglets, reproductive disorders in pregnant sows and respiratory disease complex in older pigs (Plowright et al., 1976). PCMV, an enveloped DNA virus, is classified as *Suid herpesvirus 2*, unassigned species in the subfamily *Betaherpesvirinae* (Pellett et al., 2012). It has been shown that latent PCMV infection can be reactivated when pigs are stressed (Edington et al., 1976). An immunosuppressive potential of PCMV is discussed; recently, Liu et al. (2014) showed on transcriptome level that PCMV infection alters functions of immune system by regulating the expression of multiple cytokines.

PCMV is a pathogen typically transmitted oronasally. The second possible route of transmission is congenital. The virus is shed in nasal secretions and it has also been detected in ocular secretion, urine, cervical fluid and semen (Liu et al., 2013). PCMV infects mainly mucosal glands, lacrimal glands and kidney tubules. Replication of the virus was described also in the capillary endothelium and sinusoids of lymphatic tissues in foetal and neonatal pigs. Watt et al. (1973) found that porcine alveolar macrophages (PAMs) were highly sensitive for primary isolation and propagation of the virus. Presence of PCMV in these cells was proved by electron microscopy (Valicek and Smid, 1979). PCMV DNA was also detected in porcine monocytes/macrophages by Guedes et al. (2004).

Based on this data it seems evident that macrophages are the appropriate cell type for study of PCMV immunobiology. The goal of this study was to set up *in vitro* model of PCMV infection in monocyte derived macrophages (MDMs) and investigate whether the infection alters cytokine expression of these antigen presenting cells (APC).

2. Material and methods

2.1. Virus propagation on PT-K75 cells

The Kanitz strain of PCMV (ATCC® VR-1499™) was purchased from the American Type Culture Collection (ATCC). The virus was propagated on the PT-K75 cell line (ATCC® CRL-2528™) of porcine (*Sus scrofa*) nasal turbinate origin and maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Biosera) supplemented with 20% foetal bovine serum (FBS) (GE Healthcare Life Sciences) and 1% L-glutamine (100x; Biosera) at 37 °C and 5% CO_2 for 7 days according to supplier's instructions with minor modification (FBS concentration was increased

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from 10% up to 20%). The virus stock was clarified by centrifugation (4000*g*, 30 min) to remove cell debris, aliquoted, and stored at −80 °C until further use. For the purpose of virus quantification, real-time PCR (qPCR) was used. The propagation of PCMV on PT-K75 cell monolayer was measured in 24-well polystyrene cell-culture plates (TPP). The confluent monolayer of PT-K75 cells was infected with PCMV (1.6×10^6 DNA copy number per well) and incubated for 2 h. Monolayer was washed with phosphate-buffered saline (DPBS) (Biosera) to remove non-adsorbed virus, and complete medium was added. The cells were incubated at 37 °C in 5% CO_2 during the experiment. The infected cells were frozen (−80 °C) to release virus at 1, 3, 4, 5, 7, 9, 10, 11, 13, and 14 DPI (days post-infection), and the virus concentration was determined by qPCR.

2.2. PCMV quantification by qPCR

DNA from cell cultures was extracted with the QIAamp DNA Blood Mini Kit (Qiagen), and the quantification of PCMV genomic DNA was carried out with the use of qPCR assay (KAPA Probe Fast Universal qPCR Kit, Kapa Biosystems). Cycle parameters were as follows: initial cycle of denaturation (95 °C/10 min), 45 cycles of PCR (95 °C/15 s, 60 °C/1 min). Standard curve for the absolute quantification of DNA in samples was prepared with the serial dilution of plasmid DNA containing specific PCR product of interest (TOPO TA Cloning kit, Invitrogen). Primers and probe used for qPCR target the gene for PCMV DNA polymerase (PCMVpolF − 5′-GCTGCCGTGTCTCCCTCTAG-3′, PCMVpolR − 5′-ATTGTTGATAAAGTCACTCGTCTGC-3′, probe − 5′-FAM-CCATCACCAGCATAGGGCGGGAC-BHQ-3′). Detected PCR amplicon was 82 bp (Fryer et al., 2004).

2.3. Preparation of MDMs/PAMs

MDMs were prepared as described previously by Kavanova et al. (2017). Briefly, blood was collected from 5-month-old pigs and peripheral blood mononuclear cells (PBMC) were isolated by gradient centrifugation (830*g*, 40 min) using Histopaque-1077 (Sigma-Aldrich). CD14+ cells were isolated by positive magnetic bead selection with LS separation columns (Miltenyi Biotec). Isolated monocytes were cultured in 24-well plates at a concentration of 5×10^5 cells per well in 1 mL of DMEM supplemented with 10% FBS and 1% antibiotics (Antibiotic Antimycotic Solution 100×: 10,000 units penicillin, 10 mg streptomycin, and 25 µg amphotericin B per mL; Sigma-Aldrich) and incubated for six days at 37 °C in 5% CO2 to differentiate into macrophages.

PAMs were obtained by bronchoalveolar lavage, thereafter macrophages were stored in liquid nitrogen until use and then cells were prepared as described previously (Kavanova et al., 2015). Viability of cells was checked by trypan blue exclusion after thaw process and then PAMs were placed into 24-well plates at the same concentration as MDMs in complete medium.

The use of animals was approved by the Branch Commission for Animal Welfare of Ministry of Agriculture of the Czech Republic (approval protocol No. 14108/2015-MZE-17214) as a part of project Novipig (QJ1510108).

2.4. Quantification of PCMV multiplication in PAMs/MDMs

Prepared macrophages were infected with PCMV (1.6×10^6 DNA copy number per well) at 24 h after seeding (PAMs) or immediately after differentiation (MDMs). Macrophages were washed to remove non-adsorbed virus, and DMEM with 10% FBS and 1% antibiotics was added two hours post infection (PI) with PCMV. Macrophages were incubated at 37 °C in 5% CO_2 during the whole experiment. The infected cells were frozen (−80 °C) to release virus at 0, 3, 6, 10, 12 and 14 days post infection (DPI), and the virus concentration was determined by qPCR.

2.5. MDM expression of immune relevant genes—experimental design and sampling

Prepared MDMs were infected with PCMV (1.6×10^6 DNA copy number per well) immediately after differentiation. The medium from uninfected PT-K75 cells was used as mock-infection, and complete medium alone was used as a control. MDMs were washed to remove virus, and DMEM with 10% FBS and 1% antibiotics was added two hours PI. The following groups were included in the trial: PCMV infected cells, mock infected cells and non-infected control. The culture supernatants were collected at 1, 6 and 11 DPI for the evaluation of cell mortality. Mortality of infected cells was detected using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega) following the manufacturer's instructions.

Total RNA was extracted from harvested cells at the same experimental time points. Five independent experiments including culture duplicates were conducted, using MDMs isolated from five pigs.

2.6. Real-time RT-PCR for the detection of cytokine mRNA

Immuno-relevant gene expression (IL-8, TNF-α, IL-1β, IL-10) was quantified by real-time RT-PCR (RT-qPCR). Total RNA was isolated using RNeasy Mini Kit (Qiagen) and immediately reverse transcribed using M-MLV reverse transcriptase (Invitrogen) and oligo-dT primers (Generi Biotech). RT-qPCR was performed in 384-well-plate format, on a LightCycler 480 II (Roche), using QuantiTect SYBR Green PCR Kit (Qiagen) and gene specific primers (Generi Biotech) adopted from Kavanova et al. (2015) (Table 1).

The most stable house-keeping gene in our samples was identified using RefFinder tool (http://www.leonxie.com/ referencegene.php) and the tested genes were: Hypoxanthine phosphoribosyltransferase (HPRT), TATA binding protein 1, Hydoxymethylbilane synthase. HPRT was evaluated as the most constitutively expressed gene. Cycle parameters were as follows: denaturation (95 °C for 15 min) and 45 amplification cycles (95 °C for 15 s, 58 °C for 30 s and 72 °C for 30 s). The threshold cycle values (Ct) were determined and normalized to the Ct value of HPRT reference mRNA (ΔCt). Normalized mRNA levels were calculated as $2(-\Delta Ct)$ (Livak and Schmittgen, 2001). Results are expressed as relative expression of mRNA between the treatment and control.

2.7. Statistical analysis

The normality of data distribution was tested by the Shapiro-Wilk's W-test, and homogeneity of variances by the Levene's test. Experimental groups were compared using Wilcoxon signed-rank test, a non-parametric test for paired samples (Statistica 12; StatSoft). Differences were considered significant if $p < 0.05$.

Table 1
List of primers used for RT-qPCR quantification of gene expression.

	Sequence 5′-3′
HPRT-For	GAG CTA CTG TAA TGA CCA GTC AAC G
HPRT-Rev	CCA GTG TCA ATT ATA TCT TCA ACA ATC AA
IL-1β-For	ATG CTG AAG GCT CTC CAC CTC
IL-1β-Rev	TTG TTG CTA TCA TCT CCT TGC AC
IL-8-For	TTC TGC AGC TCT CTG TGA GGC
IL-8-Rev	GGT GGA AAG GTG TGG AAT GC
IL-10-For	TGA AGA GTG CCT TTA GCA AGC TC
IL-10-Rev	CTC ATC TTC ATC GTC ATG TAG GC
TNFα-For	CCC CCA GAA GGA AGA GTT TC
TNFα-Rev	CGG GCT TAT CTG AGG TTT GA