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Research paper

Comparative phenotypic and functional analyses of the effects of autologous plasma and recombinant human macrophage-colony stimulating factor (M-CSF) on porcine monocyte to macrophage differentiation

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ABSTRACT

Porcine monocyte-derived macrophages (moMΦ) have been employed as a model cell in numerous studies of the porcine immune system. However, the lack of a standardized method for moMΦ differentiation hampers the comparison of results coming from the use of different laboratory protocols. In this study we compared the use of varying concentrations of autologous plasma (10, 20 and 30% v/v) or recombinant human macrophage-colony stimulating factor (hM-CSF; 50, 100, and 200 ng/ml) to differentiate porcine monocytes into macrophages. Changes in cell morphology and surface marker expression were assessed by confocal microscopy and flow cytometry. Macrophage differentiation was evaluated by analysing TNF-α response to LPS stimulation and determining cytokine secretion patterns under both basal conditions and after classical and alternative activation. The effects of the differentiation methods on metabolic activity and susceptibility to infection with the myelotropic African swine fever virus (ASFV) were also evaluated. Monocytes cultured using the different culture conditions tested augmented in dimension and cellular complexity, but increasing porcine plasma concentrations resulted in a dose dependent enhancement in granularity and a marked pleomorphism. As expected, CD163, MHC class II DR and CD203a expression were up-regulated in both hM-CSF (M-CSF-moMΦ) and autologous plasma cultured macrophages (AP-moMΦ), although a lower percentage of $CD163^+$ cells were found following differentiation with high percentages of porcine plasma. We observed enhanced number of viable cells using high concentration of hM-CSF compared to porcine plasma, suggesting a proliferative effect. Irrespective of differentiation conditions, monocyte differentiation into macrophages resulted in an increased susceptibility to ASFV and yielded larger amounts of LPS-induced TNF-α. AP-moMΦ showed a higher basal release of IL-1RA compared to those cultured with hM-CSF and displayed a reduced ability to respond to classical activation, suggesting that the use of high percentages of porcine plasma led to the acquisition of a M2-like phenotype. We conclude that all the protocols tested in this study can be considered as suitable to produce porcine moMΦ, although the use of hM-CSF provides high responsiveness to M1 polarization. Since a higher phenotypic and functional inter-animal variability was observed in AP-moMΦ, we propose that the use of low concentration of hM-CSF should be adopted as the method of choice to provide a better reproducibility between experiments.

Abbreviations: ASFV, African swine fever virus; hM-CSF, human macrophage colony stimulating factor; moMΦ, monocyte-derived macrophages; moM2, Monocytes derived alternative activated macrophages; moM1, monocytes derived classical activated macrophages; AP moMΦ, moMΦ differentiated using autologous plasma; M-CSF moMΦ, moMΦdifferentiated using hM-CSF

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1. Introduction

Monocytes are primary immune cells, which derive from myeloid progenitors and are released from the bone marrow into peripheral blood (Volkman and Gowans, 1965; Gordon and Taylor, 2005). Circulating monocytes migrate into tissues in both the steady state and in response to inflammation, where they differentiate into macrophages or into specialised cells such as dendritic cells and osteoclasts (Volkman and Gowans, 1965; Mosser and Edwards, 2008). Macrophages maintain tissue homeostasis; playing a major role in the clearance of senescent cells and in the repair and remodelling of tissues after inflammation (Perry and Gordon, 1988). Macrophages can also rapidly respond to endogenous and exogenous danger signals generated following injury or infection and play a fundamental role in innate immune defence against pathogens (Martinez et al., 2009). Macrophage activation results in their polarization into functionally specialised subsets defined as 'classically' or 'alternatively' activated macrophages, also known as M1 and M2 cells, respectively. Classical activation by IFN-γ and LPS results a M1 phenotype characterised by secretion of high levels of pro-inflammatory cytokines and increased microbicidal or tumoricidal capacity (Mosser, 2003), whereas alternative activation by IL-4 produces a M2 phenotype primarily associated with mechanisms of immunosuppression and wound repair (Gordon, 2003; Mosser and Edwards, 2008). M2 macrophages have been recently classified into M2a, M2b, and M2c subdivisions based on the activation stimuli and transcriptional changes (Rőszer, 2015).

Given the remarkable heterogeneity and plasticity displayed by monocytes and macrophages, the use of *in vitro* models enables investigation of the phenotypic and functional changes that occur in response to environmental stimuli including pathogen challenge. Porcine monocyte-derived macrophages (moMΦ) have been used as an *in vitro* model to investigate host cell interactions with a number of important myelotropic viruses such African swine fever (ASFV) and porcine reproductive and respiratory syndrome viruses (Thacker et al., 1998; Sánchez-Torres et al., 2003; Gil et al., 2003, 2008; Vincent et al., 2005; Fishbourne et al., 2013; Garcia-Nicolas et al., 2014; Singleton et al., 2016). However, to date there is no standardized protocol for the *in vitro* differentiation of porcine moMΦ. Studies have reported moMΦ differentiation through culture with 10% foetal bovine serum (FBS) (Gil et al., 2003, 2008) or with different concentrations of porcine serum or plasma (10%: Garcia-Nicolas et al., 2014; Kyrova et al., 2014; 20%: Chamorro et al., 2000, 2004; Sánchez-Torres et al., 2003; 30%: McCullough et al., 1997, 1999; Basta et al., 1999, 2001; Tsai et al., 2010; Fishbourne et al., 2013). However, the use of FBS raised some critical issues due to the mild phenotypic changes in monocytes cultured for 7 days compared to cells cultured in porcine plasma supplemented media, whose morphological and functional features appeared very close to mature macrophages (McCullough et al., 1999). More recent studies have demonstrated the efficacy of recombinant hM-CSF in the differentiation of porcine moMΦ (Kapetanovic et al., 2013; Fairbairn et al., 2013; Singleton et al., 2016). Macrophage colony stimulating factor (M-CSF), also known as CSF-1, controls the survival, proliferation and differentiation of mononuclear phagocytes (Stanley et al., 1997), and its addition to culture media is a well-established method to efficiently *in vitro* differentiate human monocytes into macrophages (Becker et al., 1987).

To our knowledge no systematic comparison between these different methods have been published. We therefore sought to determine the optimal method for porcine moMΦ generation by directly comparing the phenotype and function following differentiation induced by varying concentrations of autologous plasma or recombinant human M-CSF.

2. Materials and methods

2.1. Animals and blood sampling

Blood samples were obtained from 6 to 18 month old healthy crossbred pigs housed at the experimental facility of Istituto Zooprofilattico Sperimentale della Sardegna (Sassari, Italy), authorized for animal research by Italian Ministry of Health. The ASFV negative status of the animals was confirmed by a commercial ELISA test (Ingenasa, Madrid, Spain) and by an immunoblotting test (OIE, 2012). Heparinized blood was collected by cranial vena cava puncture using a 50 ml syringe containing 5000 IU of sodium heparin (Thermo Fisher, Germany), connected to a mm 14-gauge needle (Delta Med, Mantova, Italy). Animal housing, handling and sampling procedures were performed in accordance with the local ethics committee.

2.2. Enrichment of monocytes

Peripheral blood mononuclear cells (PBMC) were prepared by layering 30 ml of heparinized blood diluted 2:1 in Dulbecco's phosphate buffered saline without calcium and magnesium (PBS) over 20 ml of Histopaque-1077 (Sigma-Aldrich, St. Louis, USA) and centrifuged at 600*g* for 20 min at 4 °C without breaking (Berg et al., 2013). PBMC were collected from the plasma-Histopaque interface, washed thrice in PBS at 4 °C, pelleted and re-suspended in RPMI-1640 medium (Euroclone, Milan, Italy) supplemented with 10% FBS (Thermo Fisher Scientific, Rockford, USA), penicillin (100 U/ml) and streptomycin (100 μg/ml) (Pen Strep, Thermo Fisher Scientific, Rockford, USA) (monocyte medium). Porcine monocytes were isolated by plastic adhesion using flasks (Corning, NY, USA) pre-incubated with autologous porcine plasma, according to previous methods with slight modifications (Berg et al., 2013). In brief, autologous plasma was collected from heparinized blood by centrifugation at 700*g* for 30 min at 4 °C without breaking. Flasks were incubated for 1 h at 37 °C with 5% CO_2 with autologous plasma before removal of plasma and addition of PBMC. Flasks were incubated for 1 h and non-adherent cells were then removed by 4 washes with unsupplemented RPMI-1640 and adherent cells were incubated overnight at 37 °C with 5% CO_2 in monocyte medium. The following morning adherent cells were detached by placing the flasks on ice for 1 h, pelleted by centrifugation at 200*g* for 8 min and re-suspended in monocyte medium. Live monocytes were counted using an automated cell counter (Countess, Invitrogen, Thermo Fisher Scientific) before being seeding in 12 well plates (Sigma-Aldrich) at a concentration of 7–10 × 10^5 live cells/well. Monocytes purity was confirmed to be ≥90% as assessed by flow cytometric staining of $CD14^+$ cells as described below.

2.3. In vitro differentiation of monocyte into moMΦ and activation

Six different culture conditions were tested for moMΦ differentiation. Monocytes were cultured for 5 days at 37 °C with 5% CO_2 in medium supplemented with different concentrations of autologous porcine plasma or hM-CSF. Plasma supplemented media were composed of RPMI-1640, penicillin/streptomycin (100 U/ml/100 μg/ml) and 10%, 20% or 30% (v/v) autologous porcine plasma, whereas hM-CSF supplemented media were prepared by adding 50 ng/ml, 100 ng/ml or 200 ng/ml of recombinant human M-CSF (eBioscience, San Diego, USA) to RPMI-1640 medium with 10% FBS and antibiotics. Porcine plasma and FBS were heat-treated at 56 °C for 30 min to inactivate complement before addition to culture media.

To assess macrophage activation, moMΦ obtained using 30% autologous porcine plasma or 50 ng/ml hMCSF were both classically

and alternatively activated as described in humans and pigs (Gordon et al., 2003; Garcia-Nicolas et al., 2014; Singleton et al., 2016). For classical activation, moMΦ were cultured in monocyte medium supplemented with 100 ng/ml of recombinant porcine IFN-γ (Raybiotech Inc, Norcross, GA, USA) and 100 ng/ml of LPS (Lipopolysaccharide from Escherichia coli 0111:B4; Sigma-Aldrich) for 24 h at 37 °C in 5% CO_2, while for alternative activation, cells were stimulated with 20 ng/ml of recombinant porcine IL-4 (R & D Systems, Minneapolis, MN, USA) under the same culture conditions.

2.4. In vitro infection with ASFV

The highly virulent ASFV Sardinian field strain 22653/14 was isolated from the spleen of a naturally infected pig collected from a 2014 outbreak in the province of Cagliari (Exotic Disease Laboratory ASFV Archive, IZS of Sardinia, Sassari, Italy). This isolate is placed in the p72 genotype I and cluster within sub-group X of the B602L gene, as with the other 57 Sardinian ASFV isolates collected during 2002–2014 (Sanna et al., 2016). ASFV 22653/14 was propagated *in vitro* by inoculation of sub-confluent monolayers of porcine monocytes/macrophages for no more than six passages (Malmquist and Hay, 1960). In brief, leukocytes were cultured in RPMI-1640 medium supplemented with 20% (v/v) autologous plasma, 100 U/ml penicillin and 100 µg/ml streptomycin. After 2 days, non-adherent cells were removed and virus suspension was added to the adherent monocytes/macrophage monolayer. After 2 h incubation, RPMI-1640 medium supplemented with 1% (v/v) autologous plasma, 0.1% autologous erythrocytes, 100 U/ml penicillin and 100 µg/ml streptomycin was added. After 3 days at 37 °C in 5% CO_2, the supernatant was collected and pooled with a freeze-thawed cell lysate. The resultant pool was clarified by centrifugation at 3000*g* for 15 min, aliquoted and stored at −80 °C. Viral titre of ASFV 22653/14 was obtained by serial dilution of the virus suspension on monocyte/macrophages and cell cultures were observed for hemadsorption, as previously described (Malmquist and Hay, 1960).

Monocytes and macrophages were infected using a multiplicity of infection (MOI) of 1 and mock-infected controls were included in each experiment. Before infection, culture medium was removed and infected cells were cultured in fresh monocyte medium free of plasma or hM-CSF. Twenty hours post-infection cells were harvested and infection was confirmed by intracytoplasmic detection of the late viral protein p72 using flow cytometry, as described below.

2.5. Cell morphology

Monocyte and moMΦ morphology was analysed by confocal microscopy, as previously described (Kyrova et al., 2014). Monocytes were cultured on two-well chamber slides (Thermo Fisher Scientific) at a concentration of 5×10^5 live cells/well and were observed immediately or following differentiation under the different culture conditions. Cells were fixed with 4% paraformaldehyde and labelled with Hoechst 33342 (Molecular Probes, Thermo Fisher Scientific) for nuclear staining and Alexa Fluor 488 conjugated phalloidin (Molecular Probes, Thermo Fisher Scientific) to visualize actin cytoskeleton. Microscopy was performed using a Leica SP5 Confocal Microscope (Leica Microsystem, Wetzlar, Germany) equipped with a 40X Plan-Apo 1.25 NA oil immersion objective. Images were acquired on a format of 1024 × 1024 pixel, with a line average of 2 and scan speed of 100 Hz. Images were processed with LAS AF Lite software (Leica Microsystem) for contrast and brightness adjustments. Manipulations did not change the data content.

2.6. Flow cytometry

Expression of surface markers on porcine monocytes and moMΦ differentiated using the different protocols were analysed by multiparameter flow cytometry. Cells were harvested with ice-cold PBS with 10 mM EDTA, washed in PBS and transferred to a round-bottom 96 well plate ($1–2 \times 10^5$/well) for staining. To assess viability, cells were stained with LIVE/DEAD® Fixable Far Red Dead Cell Stain Kit (Thermo Fisher Scientific) for 30 min at 4 °C and washed twice with PBS supplemented with 2% FBS. Direct and indirect staining methods based on both unconjugated and fluorochrome-conjugated mAbs specific for cell surface markers were performed. The mAbs used were: MHC class II DR (clone 2E9/13, Bio-Rad, Oxford, UK), CD163-RPE (clone 2A10/11, Bio-Rad), CD203A (clone PM18-7, Bio-Rad), CD14-PerCP and CD14-FITC (clone Tük4, Miltenyi Biotec, Bergisch Gladbach, Germany), CD16-RPE (clone G7, Thermo Scientific Pierce, Rockford, IL, USA). Primary mAbs were added to each well and incubated for 10 min at room temperature (RT). For indirect staining, secondary antibodies (Rat anti-mouse IgG2b-RPE-R & D Systems; Goat anti-mouse IgG Fc cross adsorbed RPE-conjugated, Thermo Fisher Scientific) specific for the corresponding unconjugated primary antibody were used and allowed to incubate at RT for additional 10 min after a washing step. To complete either direct or indirect immunofluorescence procedures, excess of primary or secondary antibodies were washed away with PBS. For control staining, the following irrelevant isotype-matched control antibodies were employed: mouse IgG1 isotype control PE conjugated, clone ZX3, (Thermo Fisher Scientific), mouse IgG1 negative control purified, mouse IgG2b negative control purified (Bio-Rad). To assess ASFV infection, intracytoplasmic staining was performed. Cells were fixed and permeabilized using Leucoperm (Bio-Rad) according to manufacturer's suggestion. Following permeabilization, cells were incubated at RT for 30 min with a FITC-conjugated monoclonal antibody specific for ASFV late viral protein p72, (clone 18BG3, Ingenasa) and washed in PBS. Cells were re-suspended in PBS, transferred into 12 × 75 mm round bottom tubes and at least 5000 live monocytes/moMΦ were acquired on a FACS Calibur (FACS Calibur, BD, Franklin Lakes, USA) flow cytometer and analysed using Cell Quest Pro software (BD). Analysis of data was performed by gating on viable cells (Live/Dead Fixable Dead Cell Stain negative) in the monocyte/macrophage population, and their expression of surface and intracytoplasmic markers was assessed. Gates for surface markers were set using the corresponding isotype controls, whereas gates for p72 were set using the mock-infected controls.

2.7. Assessment of metabolically active cells

A colorimetric assay based on the reduction of the MTS tetrazolium salt was used to assess metabolically active cells (CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay; Promega, Madison, USA). Monocytes were seeded into a 96 well plates at a concentration of $1–2 \times 10^5$ live cells/well and differentiated into moMΦ with the six different methods as mentioned above. A control sample prepared with monocytes cultured in RPMI-1640 medium with 10% FBS and antibiotics was also included. After 3 days, medium was discarded, cells were washed twice with RPMI-1640 and 100 µl/well of fresh monocyte medium were added. The assay was performed, according to manufacturer's instruction, and absorbance was read with an Epoch microplate reader (BioTek, Winoosky, VT, USA).

2.8. TNF-α release in response to LPS stimulation

Analysis of TNF-α secretion from monocytes and moMΦ in response to LPS stimulation was also performed. Briefly, culture media were discarded and replaced with fresh monocyte medium, then cells were stimulated with 100 ng/ml of LPS. Unstimulated cells were used as a negative control. After 6 h of culture, supernatants were collected, cleared by centrifugation at 2000*g* for 3 min and stored at −80 °C until analysed. The measurement of TNF-α levels in culture supernatants was performed using a Porcine TNF-α Duoset ELISA (R & D System), according to the manufacturer's instructions and absorbance was read with an Epoch microplate reader (BioTek).

2.9. Patterns of cytokine secretion

Analysis of basal cytokine release from moMΦ differentiated in different media and in response to classical and alternative activation was performed using a multiplex immunoassay. Monocytes were seeded in 12 well plates and differentiated into moMΦ under the different conditions as described above. After 5 days, supernatants were collected, cleared by centrifugation at 2000*g* for 3 min and stored at −80 °C until analysed. MoMΦ cultured in 30% autologous plasma or 50 ng/ml hM-CSF were left untreated or classically and alternatively activated. After 24 h activation, supernatants were collected, cleared by centrifugation at 2000*g* for 3 min and stored at −80 °C until analysed. The simultaneous measurement of IL-1α, IL-1β, IL-1RA, IL-6, IL-10, IL-12 and TNF-α was performed using the Porcine Cytokine/Chemokine Magnetic Bead Panel Quantikine kit (Merck Millipore, Darmstadt, Germany) and a Bioplex MAGPIX Multiplex Reader (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions. The intra-assay and inter-assay CV were 10% and 20%, respectively.

2.10. Data analysis and statistics

All experiments were performed in duplicates (for metabolic activity assay and multiplex cytokine immunoassay) or triplicates (all other assays), and repeated at least three times with different blood donor pigs. Graphical and statistical analysis was performed using GraphPad Prism 5.04 (GraphPad Software Inc, La Jolla, USA) and Minitab (Minitab Inc., Coventry, UK). All data were checked for normality using the Anderson Darling test and analysed by one-way analysis of variance (ANOVA) followed by a Tukey's pairwise comparison test for normal distribution or alternatively by the non-parametric Mann-Whitney test or Kruskal-Wallis followed by Dunn's multiple comparison. A p value < 0.05 was considered statistically significant.

3. Results

3.1. Phenotype characterization of moMΦ

MoMΦ differentiated by both autologous plasma (AP-moMΦ) and hM-CSF (M-CSF-moMΦ) increased significantly in dimension and cellular complexity compared to monocytes as respectively shown by their forward (FSC) and side angle (SSC) light scattering properties measured by flow cytometry (Fig. 1). No significant differences were observed between moMΦ populations in terms of FSC, instead SSC was increased in AP-moMΦ compared to M-CSF-moMΦ (Fig. 1). Increasing porcine plasma concentrations produced a dose dependent increase in granularity as shown by the SSC values. Confocal microscopy showed that AP-moMΦ appeared more pleomorphic and presented an increased number of elongated projections protruding from cell surfaces compared to M-CSF-moMΦ (Fig. 1).

We observed that moMΦ differentiation by either method induced a cell surface immunophenotype significantly dissimilar from monocytes, with a significant up-regulation in expression of CD163, MHC-II DR and CD203a (Fig. 2). CD163 was more highly expressed in M-CSF-moMΦ compared to AP-moMΦ. Percentages of MHC-II DR^+ and $CD203a^+$ cells were similar between protocols with the only exception of moMΦ differentiated with 30% of autologous plasma, which presented significantly higher proportions of $CD203a^+$ cells compared to M-CSF-moMΦ (Fig. 2). The expression of other two myeloid markers (CD14, CD16) was also assessed and no statistically significant differences between AP-moMΦ and M-CSF-moMΦ were detected (Fig. 2).

3.2. Assessment of metabolically active cells

As displayed in Fig. 3, moMΦ differentiated using hM-CSF as well as 20% and 30% of autologous porcine plasma showed enhanced metabolic activity compared to monocytes cultured in RPMI-1640 medium supplemented with only 10% FBS. M-CSF-moMΦ, and the 200 ng/ml

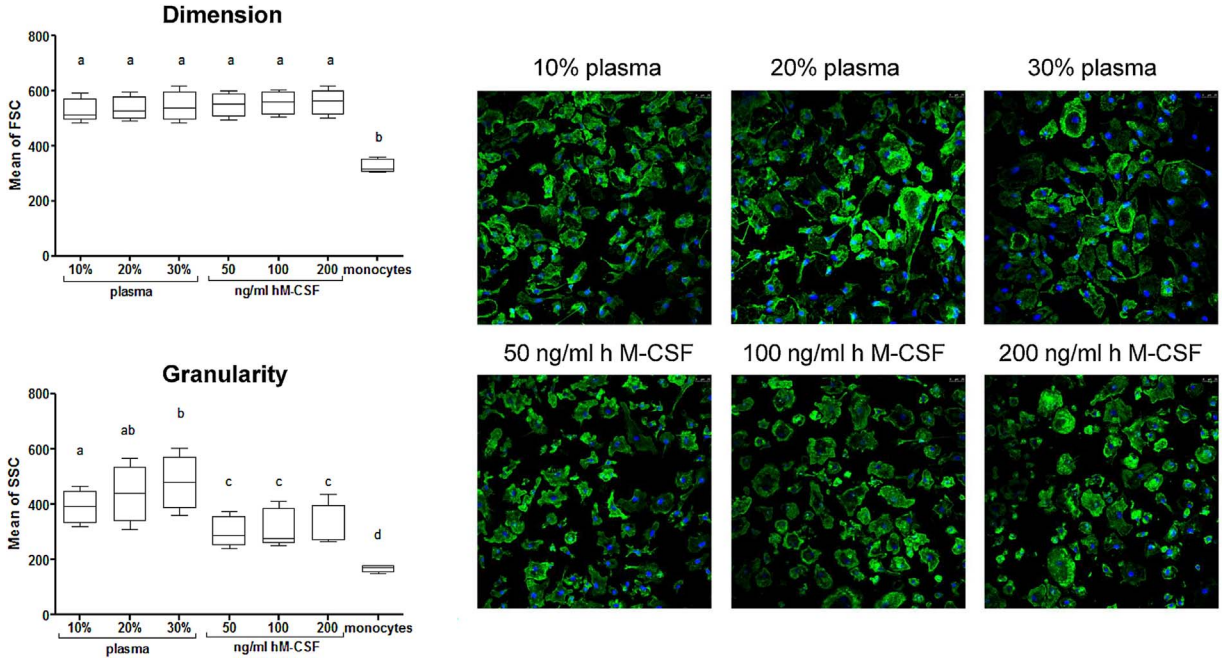

Fig. 1. Morphological analyses of monocytes and moMΦ populations. Blood derived monocytes were analysed immediately or differentiated into macrophages through incubation with RMPI-1640 supplemented with 10, 20 or 30% (v/v) of porcine plasma, or in RMPI-1640 supplemented with 10% FBS and 50, 100 or 200 ng/ml of hM-CSF, for 5 days at 37 °C in 5% CO_2. On the left: differences between populations in terms of expression of dimension (FSC, forward scatter) and complexity (SSC, side scatter) were evaluated using flow cytometry. Boxplots indicate the median (middle line), 25th and 75th percentiles (boxes), maximum and minimum (whiskers) of N positive (N+) cells of three independent experiments performed in triplicate. Values for each marker were compared using a Kruskall-Wallis followed by Dunn's multiple comparison test. Different letters indicate significant differences between moMΦ populations ($p < 0.05$). On the right: confocal microscopy observations after staining nuclei in blue with Hoechst 33342 and cytoskeleton in green with Alexa Fluor 488-conjugated Phalloidin. Magnifications are 40X.

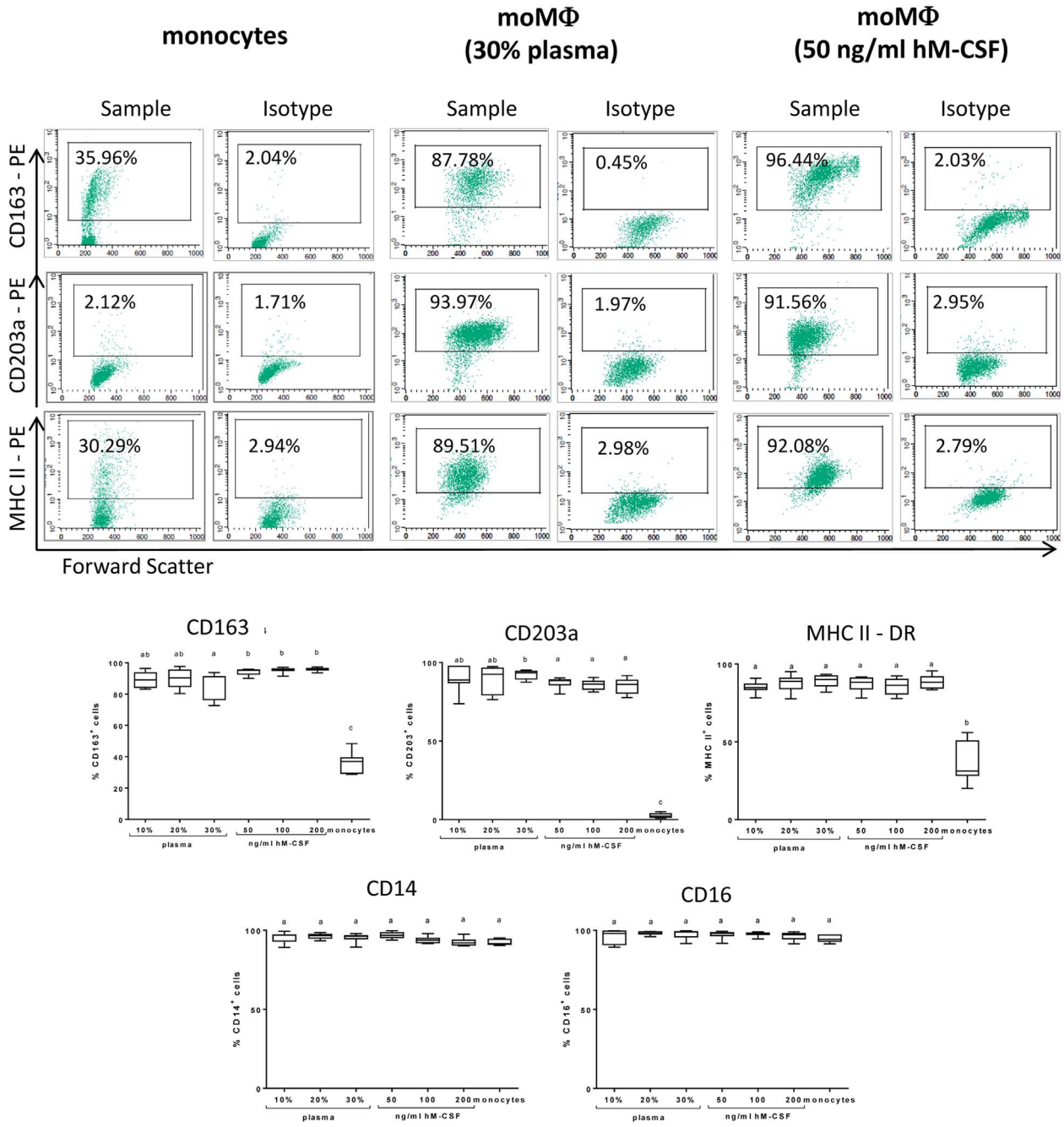

Fig. 2. Expression of cell surface markers by monocytes and moMΦ populations. Monocytes and moMΦ populations were analysed for surface markers expression and differences between monocytes and moMΦ populations in terms of CD163, MHC II DR, CD203a, CD14 and CD16 are displayed. Representative dot plots of CD163, MHC II DR, CD203a of monocytes and moMΦ differentiated using 30% of plasma or 50 ng/ml of hM-CSF are displayed. Boxplots indicate the median (middle line), 25th and 75th percentiles (boxes), maximum and minimum (whiskers) of N positive (N+) cells of three independent experiments performed in triplicate. Values of MHC II DR expression were compared using a one-way ANOVA followed by a Tukey's Multiple Comparison Test while for CD163, CD203a, CD14 and CD16 expression a Mann-Whitney Test was used. Different letters indicate significant differences between moMΦ populations ($p < 0.05$).

cultures in particular, presented increased activity compared to AP-moMΦ. Significant differences in the metabolic activity were also found between macrophages produced with 10% compared to 30% of autologous plasma.

3.3. Susceptibility to ASFV

Susceptibility to ASFV infection was assessed analysing the intracellular expression of the late viral protein p72. Our results showed that all moMΦ populations expressed p72 at higher levels compared to monocytes, with minimal differences being observed between moMΦ differentiated using the different culture conditions (Fig. 4A). Using 30% of autologous plasma a slightly lower susceptibility to ASFV infection was observed (Fig. 4A).

3.4. LPS-stimulated release of TNF-α

The ability to release TNF-α in response to LPS stimulation was also determined. As displayed in Fig. 4B, all the six moMΦ populations displayed increased TNF-α production compared to monocytes. MoMΦ differentiated using 200 ng/ml M-CSF secreted the greatest amount of TNF-α. No significant differences between those cultured in 50, 100 ng/

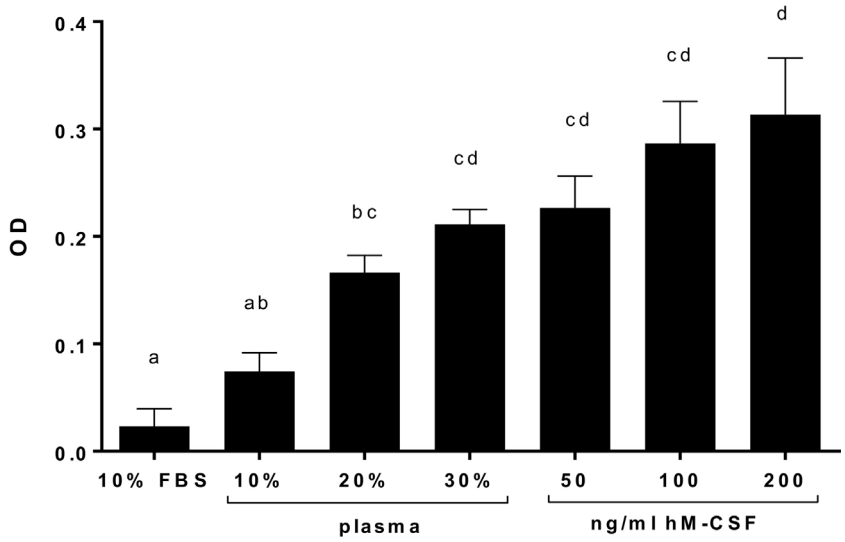

Fig. 3. Assessment of the metabolic activity of moMΦ populations. Monocytes were seeded into a 96 well plate and differentiated into moMΦ through incubation with RMPI-1640 supplemented with only 10% FBS ('10% FBS') or in RMPI-1640 supplemented with 10–20–30% (v/v) porcine plasma or RMPI-1640 supplemented with 10% FBS and 50–100–200 ng/ml of hM-CSF for 3 days at 37 °C in 5% CO_2. Metabolic activity was determined using the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay. Background absorbance was subtracted and graph represents the mean data ± SEM from three independent experiments utilizing different animals. Values were compared using a Kruskall-Wallis followed by a Dunn's multiple comparison test. Different letters indicate significant differences between moMΦ populations ($p < 0.05$).

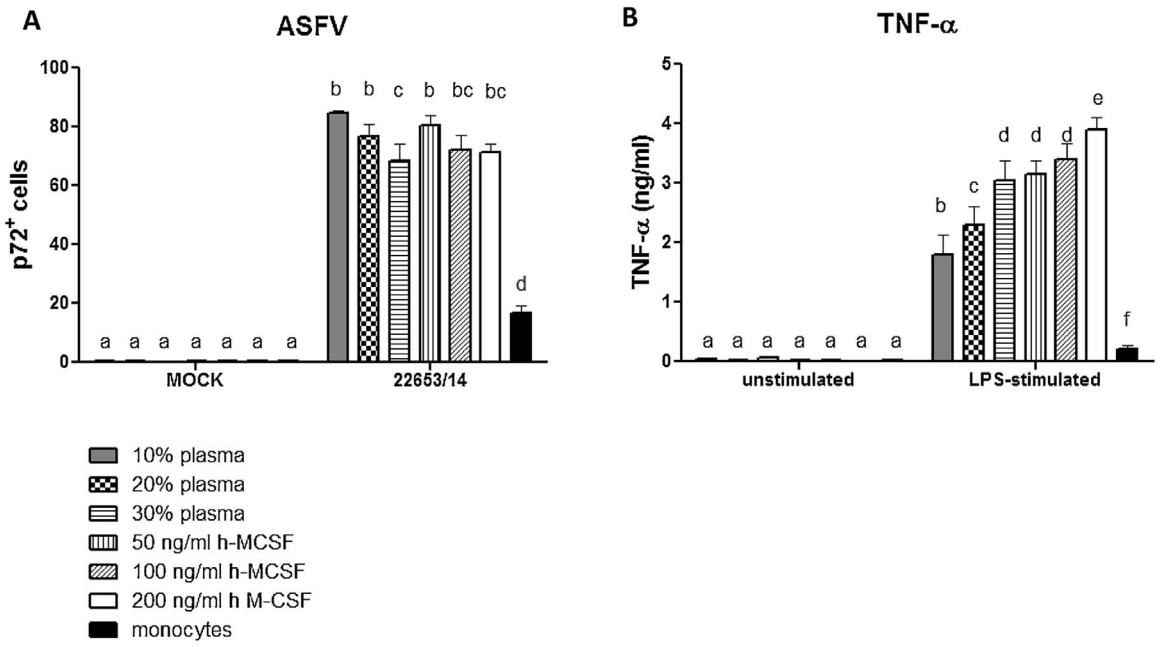

Fig. 4. Susceptibility to ASFV infection and TNF-α release in response to LPS stimulation by monocytes and moMΦ. In panel A susceptibility to ASFV is displayed. Monocytes and moMΦ populations were mock-infected or infected with the virulent ASFV isolate 22653/14 using a MOI of 1. Twenty hours post infection cells were harvested and infection was evaluated by intracytoplasmic detection of the viral p72 in flow cytometry. The mean data ± SEM from three independent experiments utilizing different animals are shown. In panel B TNF-α release in response to LPS stimulation is displayed. Monocytes and moMΦ differentiated with different methods were left untreated or stimulated with 100 ng/ml of LPS for 6 h. Supernatants were collected and stored at −80 °C till analysis. The mean data ± SEM from five independent experiments utilizing different animals are shown. TNF-α amounts in culture supernatants were evaluated using an ELISA assay. For both panels, differences between groups were compared using a Kruskall-Wallis followed by Dunn's multiple comparison test. Different letters indicate significant differences between moMΦ populations ($p < 0.05$).

ml M-CSF and 30% of porcine plasma were found. Macrophages differentiated in 10% of porcine plasma produced the lowest amount of TNF-α (Fig. 4B).

3.5. Patterns of cytokine secretion

The basal release of a panel of cytokines from moMΦ differentiated under the different culture conditions was assessed using a multiplex immunoassay. No differences in the levels of IL-1α, IL-1β, IL-6, TNF-α were observed between macrophage populations (data not shown). Instead statistically significant differences were detected in the levels of IL-10, IL-12, IL-1RA (Fig. 5). MoMΦ differentiated using 100 and 200 ng/ml hM-CSF released a statistically significantly higher amount of IL-10 compared to AP-moMΦ. Instead the supernatants of AP-moMΦ contained statistically significant higher amounts of IL-12, but this was also present in the background control (RPMI-1640 supplemented with 30% of porcine plasma), suggesting that the IL-12 was derived from the autologous plasma as opposed to being released by the AP-moMΦ. Higher levels of IL-1RA were detected in the supernatant of AP-moMΦ compared to M-CSF-moMΦ or the background control. Finally, differ-

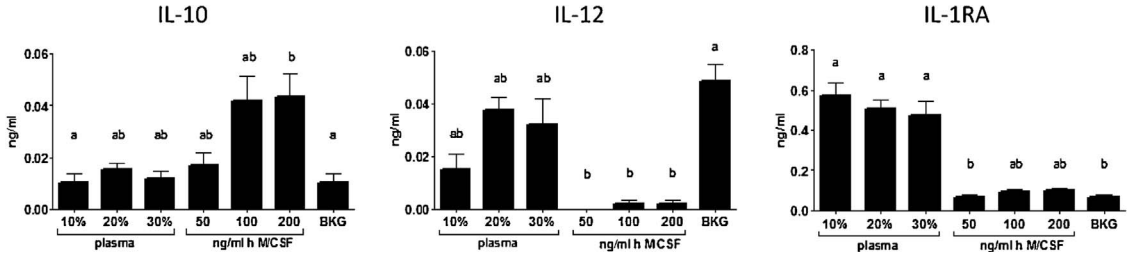

Fig. 5. Basal cytokine release from moMΦ differentiated in different culture conditions. Blood derived monocytes were differentiated into moMΦ through incubation with 10, 20 or 30% (v/v) of porcine plasma, or using 50, 100 or 200 ng/ml of hM-CSF for 5 days at 37 °C in 5% CO_2. IL-1α, IL-1β, IL-RA, IL-6, IL-10, IL-12 and TNF-α in culture supernatants and background control (BKG) were evaluated using a Porcine Cytokine/Chemokine Magnetic Bead Panel Quantikine, according to manufacturer's protocol. The mean data ± SEM from three independent experiments utilizing different animals are shown. Values for each cytokine were compared using a Kruskall-Wallis followed by Dunn's multiple comparison test. The different letters indicate significant differences between moMΦ populations ($p < 0.05$).

ences in the ability of moMΦ produced under selected differentiation culture conditions (30% of plasma and 50 ng/ml of hM-CSF) to respond to classical and alternative activation were assessed. Differentiating cells under these conditions resulted in the same levels of metabolic activity (Fig. 3) and so ensured that the number of live cells in culture would not influence the results. Culture media was discarded before macrophage stimulation. Using both protocols, classical activation resulted in release of IL-12 and pro-inflammatory cytokines IL-1α, IL-1β, IL-6, TNF-α (Fig. 6). Higher levels of IL-1α, IL-1β, TNF-α were observed in moM1 derived from 50 ng/ml M-CSF-moMΦ compared to 30% AP-moMΦ, instead there were no differences in the other cytokines tested. Almost undetectable levels of cytokines were observed after alternative activation of either moMΦ population (Fig. 6).

4. Discussion

MoMΦ have been utilised in numerous studies of the porcine immune system, especially in the context of myelotropic viruses such as ASFV and porcine reproductive and respiratory syndrome virus (Thacker et al., 1998; Sánchez-Torres et al., 2003; Vincent et al., 2005; Fishbourne et al., 2013; Garcia-Nicolas et al., 2014; Singleton et al., 2016), but currently there is no standardized protocol for their generation. The availability of a reliable standard technique would add value to efforts to both understand basic macrophage biology in the pig and to study pathogen interactions with this important immune cell population. To address this need, this study compared the most commonly described methods to differentiate porcine monocytes into macrophages *in vitro*.

Clear phenotypic differences between moMΦ produced under the different conditions were found. AP-moMΦ presented increased granularity compared to M-CSF-moMΦ, as estimated by their SSC properties in flow cytometry analysis, particularly evident when using plasma concentrations of 30%. AP-moMΦ displayed a more elongated phenotype and, as already noted by other authors (Sanchez et al., 1999), were characterized by the presence of numerous cytoplasmic projections. Using autologous plasma, a greater inter-animal variability was observed, whereas use of hM-CSF produced macrophage populations characterized by phenotypic homogeneity. Cells grown in porcine plasma or hM-CSF supplemented media presented higher dimension than those cultured in RPMI-1640 medium supplemented with only 10% FBS (data not shown), in accordance with previous publication (McCullough et al., 1999). MoMΦ differentiation is accompanied by the acquisition of new cell surface antigens, whose detection has been widely employed to identify different maturation stages within the monocyte/macrophage lineage. Previous studies reported that CD163, CD203a and MHC II-DR are up-regulated during *in vitro* differentiation of monocytes into macrophages (Kristiansen et al., 2001; Chamorro et al., 2004; Wang et al., 2011) and we observed up-regulation of these surface markers with each of the protocols tested. Nevertheless, the use of higher concentrations of porcine plasma led to a reduced expression of CD163, whereas no significant differences were observed in the levels of CD203a and MHC II-DR between moMΦ populations. In addition to a pronounced individual variability in terms of cell surface markers expression, we have also noticed a wide dispersion of data, especially evident when analysing CD163 and CD203a expression in AP-moMΦ. These evidences suggest that hM-CSF is able to initiate a

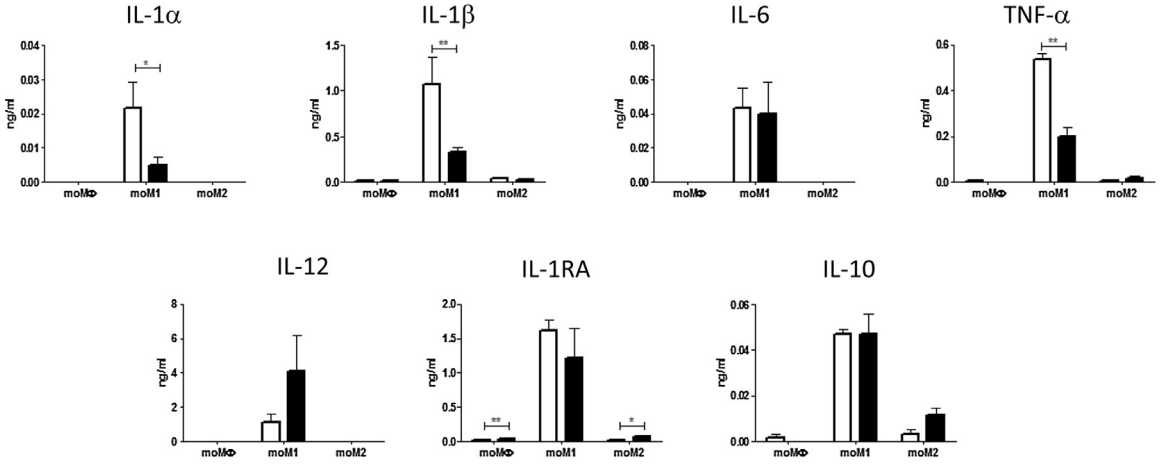

Fig. 6. Cytokines release by classically and alternatively activated moMΦ. MoMΦ differentiated through incubation with 30% (v/v) of porcine plasma (white bar) and using 50 ng/ml of hM-CSF (black bar) were both classically (moM1) and alternatively (moM2) activated. For classical activation macrophages were stimulated with 100 ng/ml of recombinant porcine IFN-γ and 100 ng/ml of LPS while for alternative activation with 20 ng/ml of recombinant porcine IL-4 for 24 h at 37 °C in 5% CO_2. IL-1α, IL-1β, IL-RA, IL-6, IL-10, IL-12 and TNF-α were detected in culture supernatants using a Porcine Cytokine/Chemokine Magnetic Bead Panel Quantikine, according to manufacturer's protocol. The mean data ± SEM from three independent experiments utilizing different animals are shown. Values for each cytokine were compared using a Mann-Whitney Test. Different letters indicate significant differences between macrophage populations ($p < 0.05$).

differentiation pathway that promotes the production of a more homogeneous cell population.

As previously reported by Wang et al. (2011), we observed that addition of both plasma and hM-CSF were able to promote porcine monocyte/macrophage survival in culture (Wang et al., 2011). In fact a lower number of cells were observed when cells were grown in RPMI-1640 medium supplemented with only 10% FBS rather than in media supplemented with porcine plasma or h-MCSF (data not show). Monocytes cultured in un-supplemented serum-free media did not survive, as previously reported in humans (Becker et al., 1897) and pigs (Basta et al., 1999). We found that supplementation with hM-CSF resulted in a higher number of metabolically active cells, particularly evident using the highest concentration tested (200 ng/ml). These results are in accordance with previous publications, in which it has been reported that, as in humans, hM-CSF acts as a mitogen inducing proliferation of pig monocytes (Fairbairn et al., 2013).

We also analysed the susceptibility of moMΦ differentiated with the different techniques to ASFV infection. The intracytoplasmic expression of late ASFV p72 protein was measured to evaluate if the differentiation method could influence viral tropism. In accordance with previous publications, moMΦ were more susceptible to ASFV infection than freshly isolated monocytes (Basta et al., 1999; McCullough et al., 1999; Sánchez-Torres et al., 2003), but we demonstrated that moMΦ showed similar permissiveness to ASFV infection notwithstanding the different media in which they were grown. MoMΦ differentiation was assessed by analysing TNF-α release in response to LPS (Gessani et al., 1993). Previous work reported that moMΦ released high levels of this cytokine in response to LPS stimulation with no differences between various pig breeds (Kapetanovic et al., 2013). We observed that macrophages differentiated using all 6 protocols released higher levels of TNF-α than freshly isolated monocytes, suggesting that with all the protocols cells acquire a macrophage-like functionality. Differences were detected between different concentrations of porcine plasma (10 *vs* 20 *vs* 30%) and hM-CSF (50 and 100 *vs* 200 ng/ml), nevertheless these were probably related to the higher number of viable moMΦ present in culture at time of stimulation.

Using different protocols, small differences were detected in the levels of IL-10 and IL-12 in culture supernatants, although IL-12 was also detected in the background plasma control. Despite the immunosuppressive role of IL-10, the levels detected were extremely low and they might not influence the *in vitro* responses of macrophages to external stimuli. In fact, M-CSF-moMΦ responded efficiently to classical activation. Greater differences were detected in the levels of IL-1RA; AP-moMΦ release higher levels of this cytokine although diversity amongst pigs was observed. It has been described that different populations of activated M2 can arise in response to different stimuli (Murray et al., 2014) and on this assumption M2 were subdivided into M2a, stimulated by IL4/IL13, M2b, in response to immune complexes and LPS, M2c to glucocorticoids and TGF-*β* (Edwards et al., 2006; Rőszer, 2015). In humans and mice, IL-1RA may act in combination with LPS, immune complexes and apoptotic cells to polarize macrophage activation towards a M2b phenotype (Duque and Descoteaux, 2014). Our results suggest that some plasma components induce IL1-RA release which might affect macrophage phenotype and potentially skew their response to external stimuli. Few studies have described classical and alternatively macrophage activation in pigs (Garcia-Nicolas et al., 2014; Sang et al., 2014; Singleton et al., 2016). In pigs classical activation induced release of IL-12 and inflammatory cytokines. We observed that M-CSF-moMΦ released higher levels of IL-1α, IL-1β and TNF-α compared to AP-moMΦ and in the latter a pronounced inter-animal variability in terms of IL-12 release in response to classical activation was observed. Considering the important role of moM1 in host defence to viruses, intracellular bacteria and protozoa (Mosser and Edwards, 2008), our results suggest that in studies on macrophages-intracellular pathogens interactions the use of recombinant hM-CSF for *in vitro* generation of moMΦ might be the more suitable protocol. In humans and mice exposure to IL-4 polarize cells toward an M2a phenotype, with production of high levels of IL-10, TGF-β, IL-1RA and chemokines that promotes recruitment of Th2 cells (Mosser, 2003; Duque and Descoteaux, 2014). In pigs, independently of the protocols used to mature macrophages, no release of IL-10 or IL-1RA from moM2 was detected. However, higher levels of IL-1RA were detected in the supernatant of AP-moMΦ compared to M-CSF-moMΦ or the background control.

In conclusion, data generated in this study suggest that all the protocols tested can be considered as suitable to differentiate porcine monocytes into moMΦ· However, the use of autologous porcine plasma, especially at high concentrations, yielded to a pronounced cell pleomorphism evidenced by a great number of spindle shaped cells. The use of 30% of porcine plasma produced moMΦ with basal release of IL-1RA, reduced expression of CD163, and ability to respond to classical activation, suggesting that these cells are developing toward a M2-like phenotype. On the other hand, supplementation with hM-CSF produced macrophage populations characterized by phenotypic and functional homogeneity and provided a better reproducibility between experiments. Furthermore, the use of hM-CSF provided a slightly higher responsiveness to M1 polarization, even if a little but statistically significant basal release of IL-10 was detected in M-CSF-moMΦ. We hope that the information generated from this study will encourage the adoption of low concentration of hM-CSF for porcine moMΦ generation and their application as *in vitro* models in virology and immunology research.

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