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Research paper

A new and efficient culture method for porcine bone marrow-derived M1- and M2-polarized macrophages

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ABSTRACT

Background: Macrophages play an important role in the innate immune system as part of the mononuclear phagocyte system (MPS). They have a pro-inflammatory signature (M1-polarized macrophages) or anti-inflammatory signature (M2-polarized macrophages) based on expression of surface receptors and secretion of cytokines. However, very little is known about the culture of macrophages from pigs and more specific about the M1 and M2 polarization *in vitro*.

Methods: Porcine monocytes or mononuclear bone marrow cells were used to culture M1- and M2-polarized macrophages in the presence of GM-CSF and M-CSF, respectively. Surface receptor expression was measured with flow cytometry and ELISA was used to quantify cytokine secretion in response to LPS and PAM_3CSK_4 stimulation. Human monocyte-derived macrophages were used as control.

Results: Porcine M1- and M2-polarized macrophages were cultured best using porcine GM-CSF and murine M-CSF, respectively. Cultures from bone marrow cells resulted in a higher yield M1- and M2-polarized macrophages which were better comparable to human monocyte-derived macrophages than cultures from porcine monocytes. Porcine M1-polarized macrophages displayed the characteristic fried egg shape morphology, lower CD163 expression and low IL-10 production. Porcine M2-polarized macrophages contained the spindle-like morphology, higher CD163 expression and high IL-10 production.

Conclusion: Porcine M1- and M2-polarized macrophages can be most efficiently cultured from mononuclear bone marrow cells using porcine GM-CSF and murine M-CSF. The new culture method facilitates more refined studies of porcine macrophages *in vitro,* important for both porcine and human health since pigs are increasingly used as model for translational research.

1. Introduction

Pigs are susceptible to many viral and bacterial pathogens, leading to large economic losses in the field (Ayudhya et al., 2012; Feng et al., 2014). Due to antibiotic (mis)use to treat infections, antibiotic resistance development is prevalent which also causes a potential threat for human health. In addition, pigs are increasingly chosen as an animal model for infection and inflammation studies in biomedical research (Lunney, 2007; Myers et al., 2007; Spurlock and Gabler 2008; Thacker and Janke 2008; Truty and Smoot 2008; Mehle and Doudna 2009; Wertheim et al., 2009), because domestic pigs, in contrast to mice, are closely related to humans. Whereas the immune parameters of mice resemble the human immunological parameters for less than 10%, for porcine an overlap of > 80% was found (Raes et al., 2005; Schneemann and Schoeden 2007; Meurens et al., 2012; Martinez et al., 2013). On the other hand, porcine studies are often hampered by the lack of reliable and reproducible *in vitro* culture systems of innate immune cells, including macrophages, which is the focus of this study.

Macrophages, originally referred as “the big eaters” by Elie Metchnikoff, are myeloid immune cells and classified as part of the mononuclear phagocyte system (MPS) along with monocytes and dendritic cells (van Furth and Cohn 1968; van Furth et al., 1972; Steinman and Cohn, 1973; Cavaillon, 2011; Merad et al., 2013; Mildner and Jung, 2014; Varol et al., 2015). Tissue resident macrophages, like Kupffer

Abbreviations: GM-CSF, granulocyte macrophage colony-stimulating factor; M-CSF, macrophage colony-stimulating factor; moDM, monocyte-derived macrophages; BMDM, bone marrow-derived macrophages

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cells in the liver, microglia in the brain and Langerhans cells in the skin, develop during embryonic development initially from early yolk sac progenitors and then from fetal liver hematopoietic stem cells. During adult life, the tissue macrophages derive from local proliferation of macrophages as well as from circulating monocytes that originate in bone marrow (Mosser and Edwards, 2008; Wynn et al., 2013; Epelman et al., 2014).

Macrophages perform many different immune functions and play a pivotal role during homeostasis due to their remarkable heterogeneity and plasticity (Mosser and Edwards, 2008; Gentek et al., 2014; Tarique et al., 2015). Initially, macrophages contribute to the elimination of pathogens by phagocytosis and killing of the invading microorganisms. By this, they play an important role in the first-line of defense against pathogens together with other innate immune cells, such as neutrophils, eosinophils and natural killer cells (Gordon and Taylor, 2005). In addition, macrophages play a key role in tissue repair by the removal of local pathogens and cell debris (Kono and Rock, 2008; Nathan, 2008) and by inducing wound healing (Ricardo et al., 2008; Wynn and Barron, 2010). Due to this dual function, macrophages were proposed to occur in a bipolar mode, *e.g.* with a pro-inflammatory (M1-polarized macrophages) (Mackaness, 1962) or anti-inflammatory signature (M2-polarized macrophages) (Doyle et al., 1994; Gordon, 2003; Verreck et al., 2004; Mosser and Edwards, 2008; Savage et al., 2008; Tarique et al., 2015). Because tissue-resident macrophages are difficult to get access to, most functional studies for human macrophages are performed with monocyte-derived macrophages (moDM) instead.

Human moDM M1-polarized macrophages display a fried egg-shaped morphology and arise from monocytes in response to Granulocyte Macrophage Colony Stimulating Factor (GM-CSF), while M2-polarized macrophages, cultured from monocytes with Macrophage Colony Stimulating Factor (M-CSF) are stretched, spindle-like cells (Fleetwood et al., 2007; Leidi et al., 2009; Nagelkerke et al., 2014). M1-polarized macrophages produce high levels of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 upon LPS stimulation and thereby support a T_H1 response. M2-polarized macrophages on the other hand, produce high levels of IL-10, suppress immune responses and promote the regulatory T cells response (Verreck et al., 2004; Fleetwood et al., 2007; Savage et al., 2008). In addition, M2-polarized macrophages express high levels of CD163, a scavenger receptor involved in the clearance of free hemoglobin (Buechler et al., 2000; Kristiansen et al., 2001).

Similar to human macrophages, pig macrophages are equipped with a wide range of pattern recognition receptors by which they can detect pathogen associated molecular patterns (PAMPs) on pathogens (Kapetanovic et al., 2012). Compared to human and mouse studies, there is a lack of good markers and other tools for porcine studies. Little is known so far about porcine macrophage culture, and even less about the M1 and M2 macrophage polarity. Several research groups use primary porcine macrophages isolated from the lung (Bi et al., 2014; Bin et al., 2014; Kuzemtseva et al., 2014) or liver (Kitani et al., 2014). Others have cultured porcine macrophages from bone marrow cells without any cytokines (Stepanova et al., 2012; Kyrova et al., 2014), or in the presence of human (Kapetanovic et al., 2012; Franzoni et al., 2017a,b) or murine M-CSF (Knetter et al., 2014; Chaudhuri et al., 2016). However, no uniform and efficient culture method for porcine macrophages has been described so far. Therefore, there is a clear need for a robust reproducible macrophage culturing system which can deliver well characterized M1- and/or M2-polarized porcine macrophages.

The main goal of this study is to establish such an efficient method to culture porcine macrophages with a clear M1 and M2 polarization, which can be used for *in vitro* studies. We describe a culture method in which porcine M1- and M2-polarized macrophages can be efficiently produced for porcine mononuclear bone marrow cells using porcine GM-CSF and mouse M-CSF, respectively. These porcine M1- and M2-polarized macrophages resemble the shape and several features of human moDM M1- and M2-polarized macrophages.

2. Material and methods

2.1. Cell isolation

2.1.1. Human monocytes

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats, obtained from healthy volunteers after informed consent was obtained as approved by Sanquin Blood supply medical ethical committee (Sanquin Blood Supply, Utrecht, Netherlands), by Ficoll-Paque Plus (ρ = 1.077 g/ml; GE Healthcare Bio-Science AB, Sweden) density centrifugation at 872 × *g* for 20 min at room temperature with a slow start and brake. PBMCs were resuspended to 2×10^8 cells/3 ml in RPMI-1640 (Life technologies corporation, Grand Island), supplemented with 10% FCS (Bodinco B.V., Alkmaar, The Netherlands) and 1% penicillin/streptomycin ($RPMI^{+/+}$) after washing twice with warm Dulbecco's PBS (dPBS) (pH7.4; Life technologies corporation, Grand Island). Monocytes were enriched by separating the PMBCs using hyper-osmotic Percoll (Sigma) solution (48.5% Percoll, 51.5% endotoxin-free water, 160 mM NaCl) and centrifugation at RT, 580 × *g* for 15 min, slow start and brake. The monocyte enriched fraction was resuspended to 1×10^6 cells/ml in $RPMI^{-/-}$, washing with dPBS and added to a 6-wells plate (1 ml/well) at 37 °C, 5% CO_2. After 1 h, the monocytes adhered to the plates and they were washed twice with warm dPBS to remove all non-adherent cells.

2.1.2. Porcine monocytes

Around 60 ml whole blood was taken from healthy adult pigs. PBMCs were isolated by Ficoll-Plaque Plus density and centrifugation at 872 × *g* for 20 min at RT with slow start and brake. The isolated PBMCs (approximately 30–50 × 10^7 cells) were washed twice with dPBS and were resuspended to 2×10^7 cells/ml in $RPMI^{-/-}$, after which 1 ml was added per well in a 6-wells plate. After 1 h at 37 °C, 5% CO_2 the non-adherent cells were washed away with dPBS. After this washing step, the adhered cells form approximately 70% confluency, comparable to human adhered cells at this stage of culture.

2.1.3. Porcine bone marrow cells

Bone marrow was harvested from the pelvis by bone marrow puncture, resulting in 20–50 ml bone marrow. The bone marrow was filtered through a 70 µm filter (FALCON, Life Science, USA), to remove any clots, after which the mononuclear cells were isolated by Ficoll density centrifugation 872 × *g* for 20 min at room temperature with a slow start and brake. Depending on the pig, 8–40 × 10^7 cells were collected. The mononuclear cells were washed, frozen in FCS/10% DMSO and stored in liquid nitrogen until further use (4 × 10^7 cells/ml). Cells were thawed just before culture, washed once and resuspended in 0.5 × 10^6/ml and cultured 1×10^6 cells per well in a 6-wells plate. From one vial, we obtained around 3×10^7 live cells as counted using trypan blue in the hemocytometer.

All animals were used and kept under the approval and guidelines of the animal ethical committee of the Utrecht University.

2.2. Macrophage culture and stimulation

All cell types were cultured in 2 ml $RMPI^{+/+}$, supplemented with 20 ng/ml porcine GM-CSF (pGM-CSF) (Invitrogen, Carlsbad, CA), murine M-CSF (mM-CSF) (Peprotech, The Netherlands), human GM-CSF (hGM-CSF) (Invitrogen, Carlsbad, CA), or human M-CSF (hM-CSF)) (Invitrogen, Carlsbad, CA) to differentiate the cells towards macrophages. 1 ml fresh $RPMI^{+/+}$ supplemented with the corresponding cytokine was added at day 3 and on day 6, the cells were washed and resuspended in 2 ml fresh $RPMI^{+/+}$ with the corresponding cytokines. Cells were stimulated at day 7 with 100 ng/ml LPS *Escherichia coli* (*E. coli*) O111:B4 (Invivogen, USA) or 100 ng/ml PAM_3CSK_4 (Invivogen, USA). After 24 h, the supernatant was collected and stored at −20 °C until further use. The cells were incubated with PBS/0.5 mM EDTA for 5 min, carefully scraped and collected for flow cytometry analysis.

Table 1
Antibodies used in flow cytometry.

Antigen	Conjugate	Clone	Isotype	Supplier	Dilution	Used for
Porcine SLA-II	FITC	2E9/B		Thermo Scientific	1:500	Porcine
Porcine CD163		G7	Mouse IgG1	BIO-RAD	1:500	Porcine
Human CD40		5C3	Mouse IgG1	Biolegend	1:250	Human
Human CD32		6C4	Mouse IgG2b	eBioscience	1:500	Human
Porcine SWC3a	PE	74 − 22-15	Mouse IgG1	Abcam	1:500	Porcine
Human HLA-DR		L243	Mouse IgG2a	Biolegend	1:500	Human
Human CD68		eBioY1/82a	Mouse IgG1	eBioscience	1:1000	Human
Human CD163	PerCP	eBioGH1/61	Mouse IgG1	eBioscience	1:500	Human
Human CD80		2D10.4	Mouse IgG1	eBioscience	1:1000	Human
Human CD86	PE Cy7	IT2.2	Mouse IgG2b	eBioscience	1:250	Human
Human CD11c		3.9	Mouse IgG1	eBioscience	1:1000	Human
Porcine CD80/86		ANC152.2/8H5	Mouse IgG1κ	Ancell	1:500	Porcine
Human CD16	APC	eBioCB16	Mouse IgG1	eBioscience	1:500	Human
Human CD64		5C3	Mouse IgG1	eBioscience	1:500	Human
Human CD14	PB	TÜK4	Mouse IgG2b	BIO-RAD	1:100	Porcine
Human CD14		HCD14	Mouse IgG2a	Biolegend	1:1000	Human
Human CD11b	APC Cy7	ICRF44	Rat IgG2b	Biolegend	1:500	Human

Summary information for all monoclonal antibodies used for flow cytometry staining, including dilution, supplier, clone and relevant isotype.

2.3. Flow cytometry analysis

After detachment, the cells were collected in a 96-wells V-bottom plate and washed once with PBS/0.5% BSA. Cells were incubated with antibody panels (Table 1) for 20 min on ice. Cells were washed once, after which the cells were measured using the BD FACS Canto-II flow cytometer (BD biosciences, USA). FSC/SSC were set in the first experiment based on the cell size of culture macrophages. In the following experiments, this was not changed. Experiments were analyzed with Flowjo software V10 (FlowJo, LLC, Ashland).

2.4. ELISA

Duoset ELISA kits for porcine TNFα, IL-6, and IL-10 and Human TNFα, IL-6, and IL-10 were obtained from R&D systems (Minneapolis, MN, USA) and used according to the manufactureŕs instructions. Samples were diluted, if needed, in dPBS/1% BSA. Absorbance was measured at 450 nm using the FLUOstar Omega microplate reader (BMG Labtech GmbH) and corrected for absorbance at 570 nm. Data was analyzed using MARS data analysis software (BMG, Labtech GmbH).

2.5. Statistical analysis

Averages of results are presented +/− SEM. Statistical analysis was performed with GraphPad Prism 6.0 (San Diego, CA) software, using the two-sided student T-test. * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$; **** = $p \leq 0.0001$.

3. Results

3.1. Porcine bone marrow derived M1-shaped and M2-shaped macrophages resemble human monocyte-derived M1- and M2-polarized macrophages better than porcine monocyte-derived macrophages

The culture system for human M1- (GM-CSF) and M2-polarized macrophages (M-CSF) derived from monocytes is well established and widely used (Murray et al., 2014; Nagelkerke et al., 2014; Sierra-Filardi et al., 2014). In contrast, the cultivation of porcine macrophages is still poorly defined and to our knowledge, no proper culture method is available to culture porcine M1- and M2-polarized macrophages. Therefore, porcine macrophages were cultured from porcine monocytes (monocyte-derived macrophages: moDM) or mononuclear bone marrow cells (BMDM). As control, human moDM were cultured with human recombinant hGM-CSF or hM-CSF. Porcine recombinant GM-CSF (pGM-CSF), commercially available, was used. In addition, since porcine M-CSF is not commercially available, murine M-CSF (cross-reacting with porcine cells (Gow et al., 2012)) was used.

In order to validate the existing human macrophage culturing system, human monocytes were cultured for 7 days in the presence of hGM-CSF and hM-CSF, which resulted in approximately 60% big, granular cells, based on the forward scatter (FSC) and side scatter (SSC) (since freshly isolated lymphocytes are located around 50 K on the FSC). The M1-polarized human macrophages (hGM-CSF) were slightly bigger, and more granular than M2-polarized (hM-CSF) human macrophages. Porcine monocytes and mononuclear bone marrow cells gave rise to big, granular cells with all 4 cytokines (hGM-CSF, pGM-CSF, hM-CSF, mM-CSF), based on FSC/SSC. However, if monocytes were used as initiator cells, only a low percentage of big, granular cells was obtained, whereas the use of bone marrow cells resulted in similar percentages big, granular cells as observed with human moDM cultures (Fig. 1A).

Microscopic analysis showed the characteristic ‘fried-egg’ shape morphology for human M1-polarized moDM, while M2-polarized moDM appeared as stretched, spindle-like cells. Porcine moDM showed the typical ‘fried-egg’ shape (M1) morphology only when cultured with pGM-CSF, but not with hGM-CSF. Similarly, mM-CSF induced differentiation of porcine monocytes into the spindle like (M2) cells, but hM-CSF did not. Comparable results were observed for porcine BMDM as starting culture instead of monocytes, although some spindle-shaped cells were also observed in the culture with hM-CSF in this case (Fig. 1B). The percentage of correctly shaped macrophages is comparable to the percentage of ‘big’ granular cells found by flow cytometry. These results indicate that human cytokines were not good inducers of porcine macrophages. For clarity, porcine macrophages induced by GM-CSF will now also be referred to as M1-polarized macrophages and M-CSF induced as M2-polarized although, obviously, the validity of this actual characterization is the main goal of this study.

After the microscopic characterization of porcine and human macrophages, the expression of cellular markers was determined to further distinguish between M1 and M2 polarization. M2-polarized macrophages typically express high levels of CD163 and CD32 (FcγR-II), whereas CD64 (FcγR-I) is equally expressed on both subtypes (Nagelkerke et al., 2014). This was indeed observed for human moDM, with higher CD163 expression levels for M2-polarized moDM compared to M1-polarized moDM (Fig. 2A). Similarly, the M2 marker CD32 was higher expressed on human M2-polarized moDM, than M1-polarized moDMs (Fig. S1A). In addition, the levels of CD14 and CD16 were higher on human M2-polarized moDM (Figs. 1B and S1A). The expression of HLA-DR and CD64 were equally high on human M1- and M2-polarized moDM (Figs. 1C and S1A).

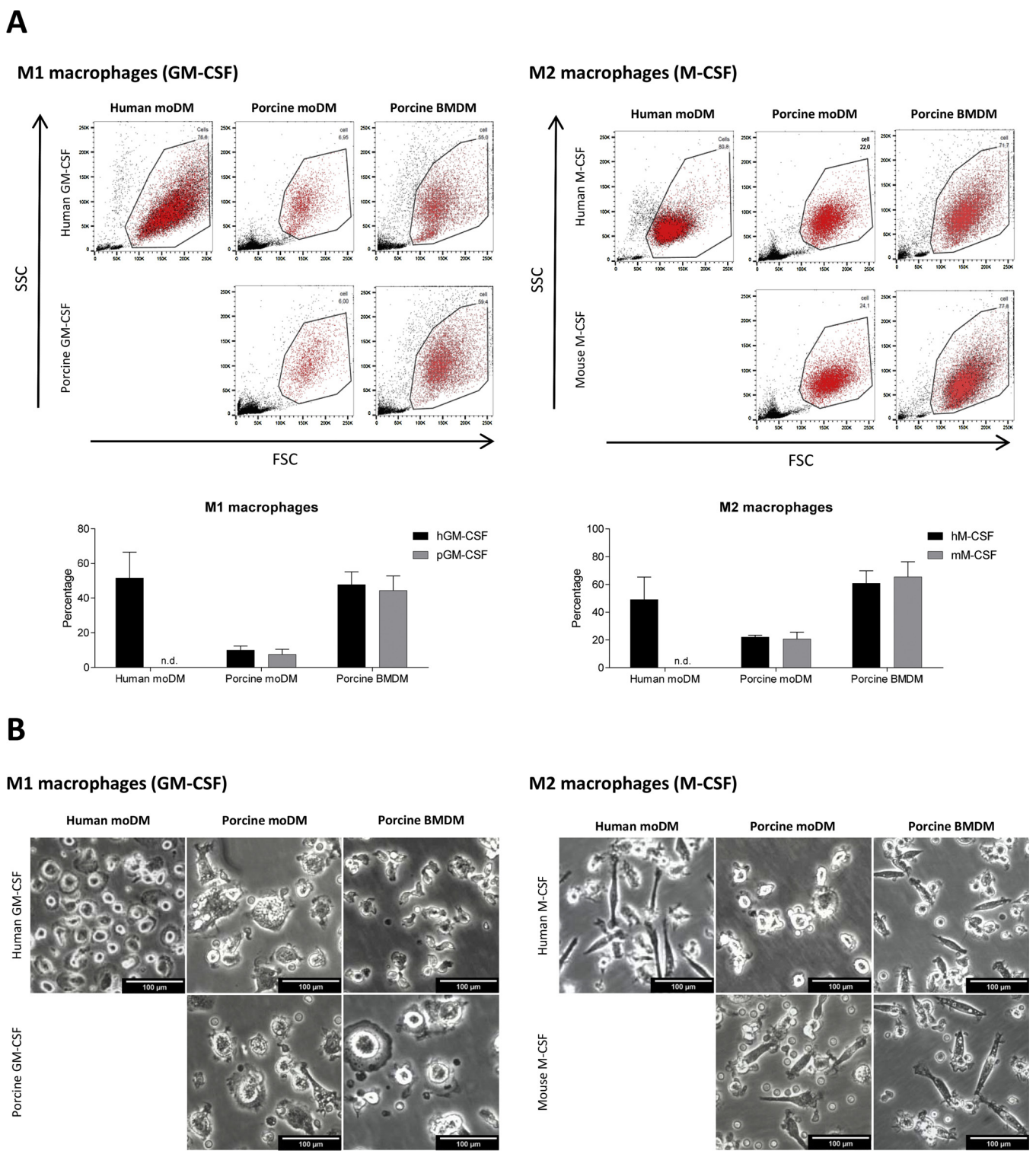

Fig. 1. The morphology of porcine moDM and BMDM macrophages, resemble human moDM.
Human or porcine peripheral blood monocytes and porcine bone marrow cells were cultured towards macrophages for 7 days in RPMI medium containing 20 ng/ml of pGM-CSF, mM-CSF, hGM-CSF or hM-CSF. Representative flow cytometry plots are depicted, with the average percentage of macrophage-like cells are based on FSC (cell size) and SSC (granularity) (A). Representative microscopic images are depicted (× 20 magnification) (B). All graphs present average ± SEM (n = 3 human moDM, n = 5 porcine moDM and BMDM).

Next, the expression of CD163, CD14, and SLA-II (porcine equivalent of HLA-DR) were measured on porcine moDM and BMDM. No differences in CD163 expression between M1- and M2-polarized porcine moDMs was observed. On the other hand, porcine BMDM followed the same expression pattern as human moDM, with a higher CD163 expression on M2-polarized BMDM compared to M1-polarized cells, which was irrespective of the origin of the M-CSF (Fig. 2A). The expression of CD14 and SLA-II on porcine moDM and BMDM did not resemble the expression pattern found in human moDM. CD14 did not show any differential expression between M1- and M2-polarized

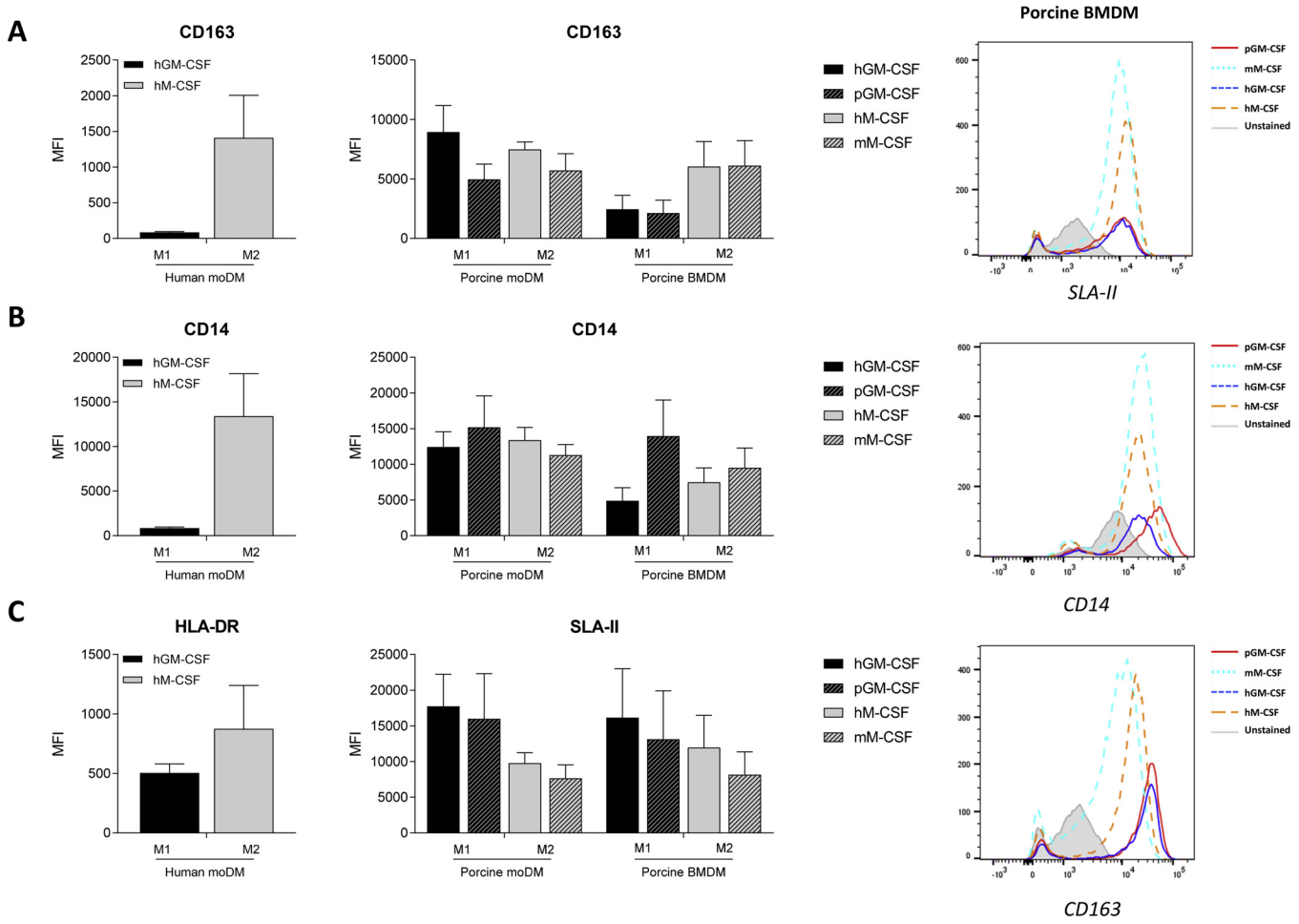

Fig. 2. Porcine macrophages closely resemble human M1- and M2-polarized macrophages based on marker expression.
Human and porcine moDM and BMDM were cultured for 7 days and subsequently were analyzed by flow cytometry. The median fluorescence intensities (MFI) of CD163 (A), CD14 (B) and HLA-DR/SLA-II (C) are depicted for human moDM, porcine moDM and BMDM. All graphs present average ± SEM (n = 3 human moDM, n = 5 porcine moDM and BMDM).

porcine macrophages (Fig. 2B). SLA-II expression was higher on M1-polarized macrophages (Fig. 2C). In the latter two markers, no difference was found between porcine moDM and BMDM.

3.2. Porcine macrophages undergo phenotypic changes upon LPS stimulation, similar to human macrophages

To further characterize the porcine macrophages and their M1/M2 polarization, the cells were stimulated with LPS. The morphology of human M2-polarized moDM remained long spindle-like shaped and obtained a lot of branches after LPS stimulation. These branches were also observed for porcine M2-polarized moDM induced by hM-CSF, although less clear. Porcine M2-polarized BMDM did not show this morphology and even lost their spindle-like shape upon LPS stimulation. Porcine M1-polarized BMDM maintained the fried egg-shape morphology also after LPS stimulation, similar to human M1-polarized moDM, induced by hGM-CSF or pGM-CSF, which was less clear for porcine moDM (Fig. 3A). In addition, the cells were stimulated with PAM_3CSK_4. The morphology changes for this stimulation were very comparable to LPS stimulation for all human and porcine macrophages, although a slight reduction in the number of branches on the spindle like cells were observed for human M2-polarized moDM (Fig. S2A).

Next, the expression of different activation markers was analyzed. Human moDM showed very little to no differences in HLA-DR upon LPS stimulation, whereas CD80 displayed a significant increase in response to LPS for both M1- and M2-polarized moDM. A similar trend for CD86 upregulation was observed for M1-, but not for M2-polarized macrophages (Fig. 3B).

In porcine macrophages, the expression of SLA-II did not change upon LPS stimulation, comparable to human moDM. As for CD80 and CD86 staining, up to date, there are no specific porcine cell antibodies commercially available for these co-stimulatory molecules. However, CD80 and CD86 can be stained simultaneously using a fusion protein antibody, CTLA-4, that binds both (Fig. 3C). In an attempt to stain CD86 more specifically, the human CD86 antibody was tested for porcine cells as well; however, no cross-reaction to porcine CD86 was found at all (Fig. 3D). PAM_3CSK_4 stimulation of macrophages resulted in small differences of activation marker expression. HLA-DR and SLA-II showed a slight decrease in both human and porcine macrophages. CD80/86 increased slightly in most samples (Fig. S2B).

3.3. Porcine M1- and M2-polarized macrophages respond similarly to their human equivalents upon stimulation

Human M1- and M2-polarized macrophages can also be distinguished by cytokine secretion, like pro-inflammatory cytokine IL-6 and TNFα (M1-polarized macrophages) and the anti-inflammatory cytokine IL-10 (M2-polarized macrophages) (Mosser and Edwards, 2008). To show that the porcine macrophages were really polarized to M1/M2 macrophages, these cytokines were measured in response to stimulation with LPS or PAM_3CSK_4.

Human M1-polarized moDM secreted high amounts of the pro-

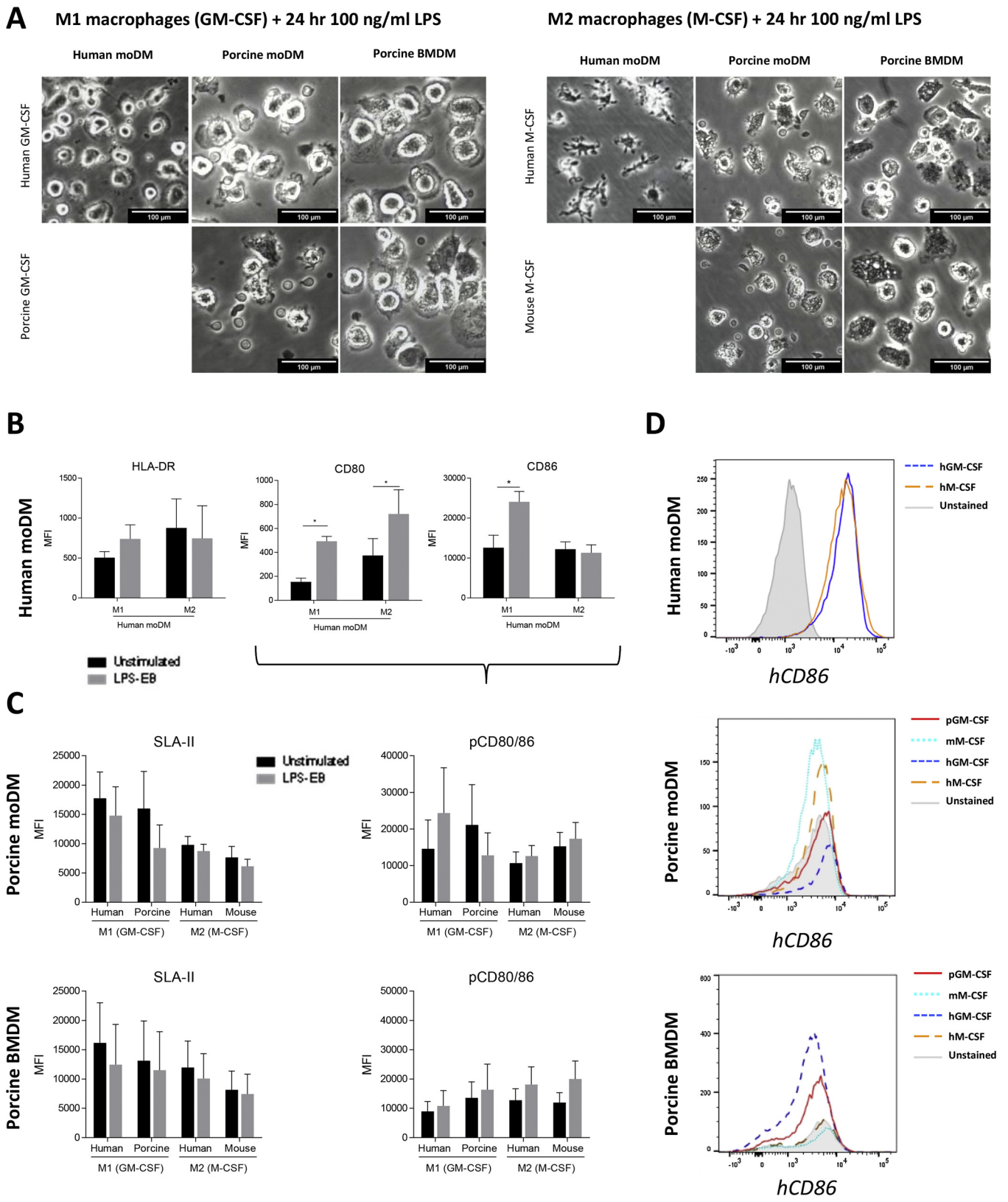

Fig. 3. Porcine macrophages undergo phenotypic changes upon LPS stimulation, similar to human macrophages.
Human and porcine moDM and BMDM stimulated at day 7 with 100 ng/ml LPS for 24 h. The morphological changes after stimulation were depicted by representative microscopic images (20 x magnification) (A). Surface marker expression in response to LPS treatment was measured by flow cytometry and depicted as median fluorescence intensity (MFI) for human moDM (B) and porcine moDM and BMDM (C). The cross-reactiveness of HsCD86 was tested on porcine cells as shown in histogram graphics (D). All graphs present average ± SEM (n = 3 human moDM, n = 5 porcine moDM and BMDM).

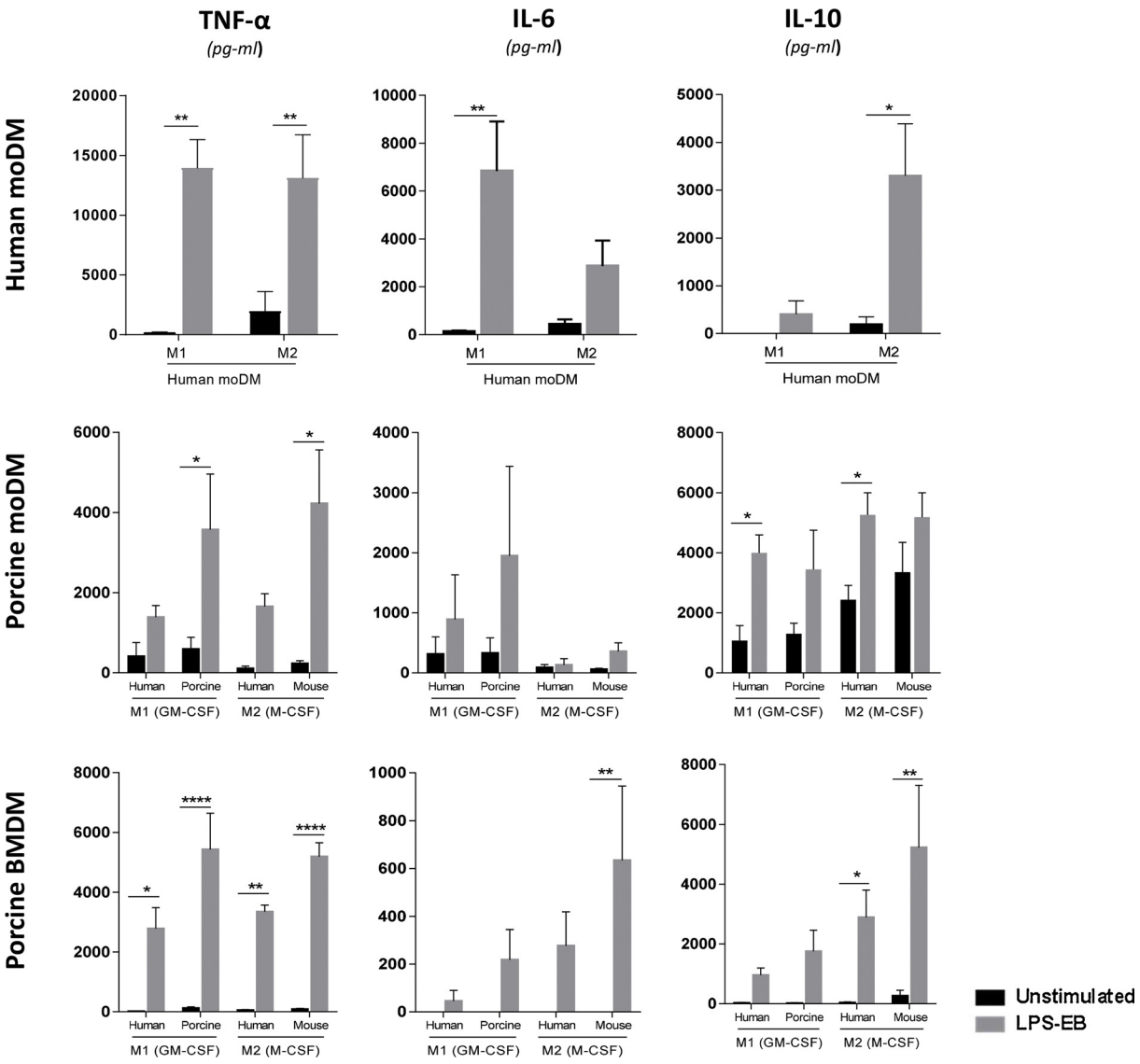

Fig. 4. Porcine M1- and M2-polarized macrophages respond similarly to their human equivalents upon LPS stimulation.
Secretion of different cytokines after stimulation with 100 ng/ml LPS for 24 h, using human or porcine specific ELISA kits. All graphs present average ± SEM (n = 4 human moDM, n = 5 porcine moDM and BMDM).

inflammatory cytokine IL-6 and low amounts of IL-10 in response to 24 h LPS stimulation. In contrast, the human M2-polarized moDM secreted low IL-6 and high amounts of IL-10 after stimulation of LPS for 24 h. Both M1- and M2-polarized macrophages secreted high amounts of TNFα upon LPS stimulation (Fig. 4). Stimulation with PAM_3CSK_4 resulted in a strong TNFα response as well by both M1- and M2-polarized macrophages. However, whereas IL-10 secretion by M2-polarized moDM was higher than by M1-polarized moDM, just like with LPS stimulation, there was no difference between M1- and M2-polarized macrophages in IL-6 secretion (Fig. S2C).

Similar to human, porcine M2-polarized macrophages secreted higher amounts of IL-10 after LPS stimulation compared to the M1-polarized macrophages, albeit larger differences were found between human and porcine BMDM. Porcine M2-polarized moDM secreted less IL-6 upon stimulation, as described in literature. Conversely, porcine M2-polarized BMDM secreted more IL-6 than M1-polarized BMDM. It should be noted that the absolute level of IL-6 secretion by porcine BMDM is much lower compared to the human moDM. The level of TNFα secretion upon stimulation was very comparable in all cultures of human and porcine macrophages. However, as observed for the morphological characterization of porcine macrophages, the use of murine and porcine cytokines instead of the human cytokines seemed to result in a higher differentiation level (Fig. 4). PAM_3CSK_4 stimulation resulted in similar IL-6, TNF-α and IL-10 responses compared to LPS stimulation. The only exception was for TNFα secretion by porcine BMDM, *i.e.* M2-polarized macrophages secreted more TNFα compared to M1-polarized macrophages (Fig. S2C).

4. Discussion

The main goal of this study was to establish a well-defined porcine macrophage culture from primary cells including the M1/M2 polarization for *in vitro* studies. Until now the lack of a proper porcine macrophage culture system forced many groups to use primary porcine macrophages isolated from the lung (Bi et al., 2014; Bin et al., 2014; Kuzemtseva et al., 2014) or liver (Kitani et al., 2014). Some culture methods for porcine macrophages have been described, derived from bone marrow cells without addition of any cytokines (Stepanova et al., 2012; Kyrova et al., 2014), in the presence of human M-CSF (Kapetanovic et al., 2012; Franzoni et al., 2017a,b) or murine M-CSF (Knetter et al., 2014; Chaudhuri et al., 2016). However, no systematic study on the use of different cytokines and different cell origins to

produce porcine macrophages and characterize them has been described so far.

Macrophages are polarized cells and can be divided into M1- and M2-polarized macrophages. M1-polarized macrophages are fried egg-shaped and produce high levels of pro-inflammatory cytokines, such as TNFα and IL-6. On the other hand, M2-polarized macrophages are stretched, spindle-like cells and produce high levels of the anti-inflammatory cytokine IL-10 (Verreck et al., 2004; Fleetwood et al., 2007; Savage et al., 2008). In addition, M2-polarized macrophages are characterized by high levels of CD163, a scavenger receptor involved in the clearance of free hemoglobin, which is not observed for M1-polarized macrophages (Buechler et al., 2000; Kristiansen et al., 2001). Due to the lack of porcine specific growth factors, different sources were tested. The obtained cell cultures were characterized based on cell morphology, known M1- and M2-polarized cell surface markers and cytokine profiles in response to bacterial ligands. Because the culture of human macrophages is widely established, using GM-CSF and M-CSF to differentiate monocytes into M1- and M2-polarized macrophages, respectively (Mackaness, 1962; Doyle et al., 1994; Gordon, 2003; Verreck et al., 2004; Mosser and Edwards, 2008; Savage et al., 2008; Murray et al., 2014; Nagelkerke et al., 2014; Sierra-Filardi et al., 2014; Tarique et al., 2015), these were used as control cells.

Human moDM indeed resulted in fried egg-shaped cells in the presence of hGM-CSF and stretched, spindle-like cells in the presence of hM-CSF. Porcine macrophages derived from either monocytes or mononuclear bone marrow cells both resembled the morphology that was found for human moDM. Although it was predicted that porcine cells could not respond to hM-CSF, but only the murine homologue (Gow et al., 2012), we did find similar morphologies for porcine macrophages using either cytokine, although differentiation of macrophages seemed less efficient with the human cytokines. The culture of porcine moDM was less efficient with only 10–20% big granular cells compared to porcine BMDM, containing up to 65% big granular cells, indicating that at first glance bone marrow is a much better source to obtain porcine macrophages.

With respect to surface markers, many similarities were observed between human and porcine M1- and M2-polarized macrophages using our culture conditions. The elevated expression of CD163 is a hallmark for M2-polarized macrophages, whereas M1-polarized macrophages express only low levels of CD163 (Nagelkerke et al., 2014). Indeed, similar to human moDM, porcine M2-polarized BMDM expressed higher CD163 levels compared to M1 BMDM, although this difference is much smaller than found for human macrophages. However, when porcine monocytes were used as starting cells, there was no clear difference in CD163 expression between (potential) M1- and M2-polarized macrophages. This indicates, together with the relatively low number of macrophages in moDM cultures, that monocytes are an inefficient source to culture porcine macrophages.

Both M1- and M2-polarized macrophages are able to respond to LPS, shown by increased expression of activation markers and cytokine secretion. M2-polarized macrophages are supposed to remain less immunogenic and therefore have less upregulation of co-stimulatory molecules and higher IL-10 secretion compared to M1-polarized macrophages (Mosser and Edwards, 2008). This was indeed observed for human moDM. Although both M1- and M2-polarized cells increased the expression of CD80 upon LPS stimulation, only M1-polarized macrophages increased the expression of CD86. For porcine cells, it was difficult to compare the results with their human counterparts, since no separate antibodies for CD80 and CD86 are available. We used a CTLA-4 labeled fusion protein antibody, which is able to bind both CD80 and CD86. Comparable to human, we observed an increase of the CD80/86 expression for porcine BMDM; however, whether the upregulation of CD86 is specific for M1-polarized macrophages is unknown. The human CD86 antibody was tested for cross-reactivity on porcine cells; however, unfortunately no cross-reactivity was found.

Upon stimulation with LPS macrophages will secrete pro- or anti-inflammatory cytokines. The cytokine expression of human moDM was as expected, with high IL-6 secretion for M1-polarized macrophages, whereas M2-polarized macrophages secreted high levels of IL-10. For porcine cells, M2-polarized BMDM indeed secreted higher levels of IL-10 compare to the M1-polarized macrophages. Unexpectedly, IL-6 expression was also higher for M2-polarized BMDM. However, it must be noted that the relative secretion of IL-6 in porcine macrophages is much lower than in human macrophages.

In summary, we describe a new and efficient method for the culture of porcine macrophages with M1- and M2-polarized polarization using porcine GM-CSF and mouse M-CSF respectively. Porcine bone marrow cells are an efficient source to culture M1-polarized and M2-polarized macrophages with many similarities to human monocyte-derived M1- and M2-polarized macrophages. The culture of porcine M1- and M2-polarized macrophages is a valuable tool for immunological studies in pig, both for veterinary use and for human studies in which the pig is used as a model system.

Declarations

All authors declare that they have no competing interests.

Authors' contributions

JG, HH and MS were responsible for the study design. JG, AD, MS performed and analyzed experiments. JG, EV, HH and MS discussed results and experiments. JG, EV and MS wrote the manuscript. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vetimm.2018.04.002.

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