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ABOUT COVER

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AIMS AND SCOPE

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Basic Study

Ex vivo effect of vascular wall stromal cells secretome on enteric ganglia

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Abstract

BACKGROUND
Mesenchymal stromal cell (MSC)-based therapy is currently under study to treat inflammatory bowel diseases. MSC bioactive products could represent a valid alternative to overcome issues associated with systemic whole-cell therapies. However, MSC anti-inflammatory mechanisms differ between rodents and humans, impairing the reliability of preclinical models.

AIM
To evaluate the effect of conditioned medium (CM) derived from porcine vascular wall MSCs (pVW-MSCs) on survival and differentiation of porcine and guinea pig enteric ganglia exposed to lipopolysaccharide (LPS).

METHODS
Primary cultures of enteric ganglia were obtained by mechanic and enzymatic digestion of ileum resections from guinea pigs (*Cavia porcellus*) (GPEG) and pigs (*Suus scrofa*) (PEG). pVW-MSCs were derived by enzymatic digestion from vascular wall resections of porcine aorta and tested by immunoflowcytometry for MSC immune profile. Enteric ganglia were treated with increasing concentrations of LPS, CM derived by pVW-MSCs or a combination of CM and LPS 1 μg/mL. Cell count and morphometric analysis of HuD positive neurons and glial fibrillary acidic protein positive glial cells were performed by immunofluorecent staining of cultured ganglia.

RESULTS
PEG showed a higher number of neurons compared to GPEG. Overall, CM exerted a protective role on LPS-treated enteric ganglia. CM in combination with

LPS increased the number of glial cells per ganglion in both cultures evoking glial cells differentiation in porcine cultures.

CONCLUSION
These findings suggest an immunomodulating activity of pVW-MSCs mediators on the enteric nervous system in inflammatory conditions.

Key words: Enteric nervous system; Mesenchymal stromal cells; Inflammatory bowel disease; Ganglia; Translational models

Core tip: Secretome of porcine vascular wall mesenchymal stromal cells (pVW-MSCs) induced an increase of glial cell number in swine and guinea pig-derived enteric ganglia. Co-treatment of enteric ganglia with lipopolysaccharide and conditioned medium promoted glial cell differentiation only in pigs. These data indicate an immune activation promoted by pVW-MSCs which could be more specific in higher mammals, suggesting a careful consideration of the animal models used in research studies on cell-based therapies.

INTRODUCTION

Inflammatory bowel diseases (IBDs), encompassing the two major forms Crohn's disease (CD) and ulcerative colitis (UC), are characterized by an overactive immune response to unknown environmental triggers associated with specific genetic traits[1]. Genome-wide association studies advanced previous knowledge of genetic variants associated with innate and adaptive immunity (*e.g.*, *NOD2, IL23R*) revealing novel pathophysiological mechanisms linked to autophagy and loss of epithelial barrier function[2,3]. In IBD, chronic intestinal inflammation induces several morpho-functional changes of the enteric nervous system (ENS), including swallowing of enteric nerve bundles and higher expression of several neurotransmitters[4].

Mesenchymal stromal cells (MSCs) are currently under study as a therapeutic option in regenerative medicine and as a novel treatment for autoimmune and chronic inflammatory disorders including IBDs[5]. Although the mechanism underlying the immunoregulatory effect of MSCs is still to be clarified, their role in balancing immune homeostasis has been acknowledged[6]. Notably, pro- or anti-inflammatory activity[7] along with other MSC biomolecules is settled by toll-like receptors 3 and 4, the latter being one of the main sensors of bacterial lipopolysaccharide (LPS)[8,9].

MSCs respond to an inflammatory environment releasing CC chemokine ligand 2 and IL-10, which inhibits CD4 Th17 cells proliferation and IL-17 production[10] and polarizes naïve T-cells to the regulatory Foxp3-positive phenotype (T-reg)[6]. These pleiotropic, anti-inflammatory properties justify a proof of concept study for a possible application of MSCs in IBDs, where Th-17 and Th-4/5 lymphocytes drive the aberrant immune reaction of CD and UC, respectively. A phase III clinical trial of CD with a systemic infusion of MSCs is currently ongoing[11], while local treatment of the severe fistulizing form of CD was recently approved by EMA[12].

One of the main drawbacks of cell-based therapy regards uncertainty about biodistribution and homing of cells to the target site of action. In particular, MSCs tend to remain trapped in the microcirculation of pulmonary alveoli, allegedly for an increased diameter acquired during *in vitro* expansion[13]. For these reasons, MSC-derived exosomes as well as MSC secretome are gaining attention in current research[14-17]. Furthermore, a recent study showed that a vascular wall mesenchymal stem cells isolated by porcine aortic tissue (pVW-MSCs) showed mesenchymal features[18] and the ability to differentiate in all the cellular components of a mature vessel[19]. A deeper characterization demonstrated their metabolic properties[20] and

their intrinsic attitude to promote angiogenesis also by paracrine action[21].

Interestingly, the key factor responsible for MSC anti-inflammatory action varies among species and is related to a specific phylogenetic tree[22]. On this basis, this study aims at investigating a possible gap between rodent and swine neuro-immune response to MSC-derived bioactive products assuming pVW-MSC secretome as a closer model from a translational point of view. To this purpose, we first compared the effect of LPS on cell survival and differentiation in primary enteric ganglia derived from guinea pig and pig myenteric plexus (MP) (GPEG and PEG, respectively); thereafter, we evaluated the effect of pVW-MSC secretome in these two *ex-vivo* models of ENS.

MATERIALS AND METHODS

Animals
Animals were used after approval of the protocol by the local ethics committee and following the guidelines of 3Rs implied in the EU directive 2010/63/EU for the use of animal for experimental purposes and in accordance with the national legislation (Decree 116/1992). In accordance with the 3Rs principle of Reduction[23] the animals used in the present study served as controls in other experimental protocols carried out in our facility.

Swine (Protocol number n.43-IX/9 all.37; 20/11/2012): Young commercial hybrids of *Sus scrofa* (4 males–aged 4-5 wk, 7 ± 0.5 Kg live weight), born at the ASA Unit (DIMEVET, University of Bologna), were enrolled in the study. Piglets were bred under the lactating sow till 28 d, then weaned and kept in a multiple box for young piglets, temperature was kept at 28 ± 1 °C with adequate ventilation and humidity in relation to the young age. Surgical procedures were carried out during the morning in the surgical theatre of the DIMEVET facilities. Animal received an i.m. bolus of tiletamine-zolazepam (5 mg/kg) 10 min before induction; general anesthesia was achieved using sevoflurane with an induction mask[24]. Animals were then sacrificed with a single bolus (0.3 mL/kg) of Tanax (embutramide/mebezonium iodide/tetracaine hydrochloride; Msd Animal Health Srl) and the abdomen was opened to remove the small intestine.

Guinea pigs (Protocol number 18/79/14): Male Dunkin-Hartley guinea pigs (*Cavia porcellus*, 8 males–aged 3-5 wk, weight 200-280 g, Harlan Italy, Udine, IT) were kept in home cages with a controlled environment (12 h dark/light cycle, 20-24 °C temperature, 40%-70% humidity) with unlimited access to water and chow. The day of the experiment, animals were sacrificed through isoflurane inhalation followed by exsanguination through jugular excision. All the procedures were carried out in the operating room of Medical and Surgical Department.

Isolation and culture of ganglia by pig and guinea pig myenteric plexus
Isolation of MP from 8 guinea pigs (3-5 wk) and 4 pigs (4-5 wk) was performed as previously described[25,26]. Briefly, the small intestine was washed with sterile, oxygenated Krebs solution containing (mM) NaCl 120.9, KCl 5, MgCl$_2$ 1.2, CaCl$_2$ 2.5, glucose 11.5, NaHCO$_3$ 14.4, NaH$_2$PO$_4$ 1.2 additioned with fungizone and penicillin-streptomycin 10 ml/L (Sigma Aldrich-Merck, Darmstadt, Germany). MP was peeled by 2-cm traits of small intestine cut in 1 mm × 1 mm fragments and digested in T25 plastic flasks with an enzymatic solution containing 1.25 mg/mL collagenase IV from *Clostridium histolyticum*, 1 mg/mL dispase II from *Bacillus polymyxa* and 1 mg/mL bovine serum albumin (Sigma Aldrich-Merck) in gentle agitation 30 min (guinea pig tissues) or 45 min (pig tissues) at 37 °C. Reaction was stopped by placing flasks in ice for 3 min. Digested tissues were washed with cold Krebs solution and collected in DMEM. Fragmented neuronal fibers were selected over muscle bundles with a stereomicroscope (Nikon C-PSCN - Nikon, Tokyo, Japan) and seeded on polyornithine-covered coverslips in 24-well plates with M199 medium enriched with 5% fetal bovine serum, 10 mL/L penicillin-streptomycin and 5% glucose (complete M199-cM199). Plates were kept 24 h in a humidified chamber at 37 °C with 5% CO$_2$.

Immune profiling and collection of media conditioned by porcine vascular wall mesenchymal stromal cells
pVW-MSCs were isolated, characterized and maintained as previously described[27]. In order to confirm the mesenchymal immunophenotype after cryopreservation, flow cytometry analysis was performed before media collection. Briefly, 2 × 10^5 cells were resuspended in 100 μL of phosphate buffered saline (PBS) and incubated for 1 h at 4 °C in the dark with appropriate fluorochrome-conjugated antibodies at the titers

reported in Table 1. Unstained controls to evaluate inherent background or autofluorescence were obtained omitting primary antibodies. After incubation, cells were washed twice and resuspended in 200 µl of PBS then analyzed with MacsQuant Analyzer10 (Miltenyi Biotec, Bergisch Gladbach, Germany). For CD34 staining, after the first incubation with the primary antibody, cells were washed and incubated with PE-conjugated secondary antibody (Table 1) for 40 min at 4 °C in the dark. Data were analyzed using the Flowlogic™ software (Miltenyi Biotec).

After thawing cellular suspensions were plated in a 24-multi well plate at a concentration of 3×10^4 cells/well in PGM medium (Promocell, Heidelberg, Germany), the day after, cells were washed with PBS and cultured for additional 24 h in PGM, then media were collected, centrifuged at $800 \times g$ for 10 min, filtered through a 0.20-µm syringe filter, immediately frozen in liquid nitrogen and stored at -80 °C until use.

Treatments

Enteric ganglia derived from each animal were seeded in 24 wells plates, a pool of 35 ganglia per well from 3 wells (triplicates) were considered for the analysis. After 2 d, ganglia were incubated for 24 h with cM199 (CRTL) or one of the followings: cM199 + 0-0.1-1-10 µg/mL LPS (LPS from Escherichia coli O111:B4, Sigma Aldrich-Merck); conditioned medium (CM) derived by culture flasks containing adherent pVW-MSCs (10% in M199) or CM combined with LPS 1 µg/mL. Treatments were coded arbitrary so that a second operator could carry on the operation blindly.

Immunocytochemistry analysis of enteric ganglia

At the end of 24-h treatment, cells were washed twice in cold PBS and fixed in 4% paraformaldehyde for 1 h. After three washes with cold PBS, unspecific epitopes were blocked by incubating fixed ganglia with a blocking solution of 0.5% Triton and donkey serum 5% for 1 h. Ganglia were double-stained by overnight incubation at 4°C with a mix containing antibodies directed to the pan-neuronal marker HuD and to the glial fibrillary acidic protein (GFAP). The following day, cells were washed three times with PBS and incubated 2 h at room temperature with appropriate fluorescent anti-antibodies (Table 1). Negative controls included a pre-adsorption step for 2 h with the specific blocking peptides in the preliminary tests and the omission of the primary antibody in every run experiment. At the end of the procedure, coverslips were mounted on slides with an anti-fade solution (10% Mowiol 4-88, Sigma Aldrich-Merck) containing 0.1 µg/mL DAPI. Photomicrographs of single ganglion were obtained with a Zeiss Imager M1 microscope with dedicated software (AxioVision, Carl Zeiss, Jena, Germany).

Imaging analysis of cultured ganglia

Cell count and morphometric analysis of photomicrographs were carried out blindly with Image J software on the basis of a previously applied method[28]. Briefly, two axis intersecting at a 90° angle were traced from the furthest ends of the cluster of cell bodies. A first circle representing the core area was traced considering the intersection of the two axis as the center and the longest axis as the diameter. Likewise, an outer circle, having the same center as the former and the diameter extending to the furthest fillopodium, was considered as the total area. The percentage of ganglion expansion (Gang. Exp. %) on total area was calculated as follows: Gang.Exp.% = [(total area-core area)]%100/(total area).

Statistical analysis

Results are reported as Tukey box-plots (middle lines-median values; lower and upper sides of the rectangles - 1st and 3rd percentile, whiskers - confidence intervals; black dots - outliers). Statistical analysis was performed through GraphPad Prism software (GraphPad, La Jolla, CA, United States) on data retrieved from 35 ganglia/well analyzed in triplicates for each experimental group. Normal distribution was confirmed by Shapiro-Wilk test and Student *t* test was used to determine statistical significance of the differences observed. Data significance was considered when $P < 0.05$ or as reported in text.

RESULTS

Comparison of ganglia derived by pig and guinea pig myenteric plexa

After 2 d of culture *in vitro*, GPEG showed a more consistent morphology and cell composition in comparison with PEG. GPEG showed a globular or bean-like shapes with a core of cell bodies and glial cells radially protruding outward (Figure 1A). Conversely, PEG were characterized by larger globular, bi- or tri-lobed shapes (Figure

Table 1 Antibody reporting									
Name	Target	Clonality	Conjugation	Research resource identifiers	Species	Supplier	Catalog number	Application	Concentration used
Anti-HuD	Hu N-terminus of human HuD	Poly	-	AB_2101223	Gt	Santa Cruz Biotechnologies	sc-5977	IC	5 µg/mL
Anti-GFAP	Hu Glial Fibrillary Acidic Protein	Mono	-	AB_10689630	Ms	BD Biosciences	561483	IC	1 µg/mL
Alexa 488	Gt IgM heavy and light chains	Poly	Alexa Fluor® 488	AB_2535792	Dk	Thermo Fisher Scientific	A-21206	IC	0.5 µg/mL
Alexa 555	Ms IgM heavy and light chains	Poly	Alexa Fluor® 555	AB_2535853	Dk	Thermo Fisher Scientific	A-21432	IC	0.5 µg/mL
Anti-CD 105	Hu CD105 (L-isoform) cell surface antigen	Mono	FITC	AB_868768	Ms	Abcam	Ab53318	FC	2 µL/10^5 cells/100 µL
Anti-CD90	Hu CD90/Thy-1 cell surface antigen	Mono	APC	AB_10677422	Ms	Abcam	Ab139364	FC	1 µL/10^5 cells/100 µL
PE anti-human CD56	Hu CD56 cell surface antigen	Mono	PE	AB_314448	Ms	Biolegend	304606	FC	2 µL/10^5 cells/100 µL
Human CD44 antibody	Hu CD44 isoforms, 80-95 Kd cell surface antigen	Mono	PerCP	AB_10645506	Rt	Biolegend	103036	FC	0.5 µL/10^5 cells/100 µL
CD34 antibody [EP373Y]	Hu CD34 cell surface antigen	Mono	-	AB_1640331	Rb	Abcam	Ab81289	FC	0.8 µL/10^5 cells/100 µL
Rabbit-PE	Rb IgG heavy and light chains	Poly	PE	AB_10680576	Gt	Abcam	Ab97070	FC	0.5 µL/10^5 cells/100 µL

FITC: Fluorescein isothiocyanate; APC: Allophycocyanin; PE: Phycoerythrin; PerCP: Peridinin-chlorophyll-protein; Ms: Mouse; Rt: Rat; Gt: Goat; Dk: Donkey; Hu: Human; GFAP: Glial fibrillary acidic protein IC: Immunocitochemistry; FC: Flowcitometry.

1E) with a number of total cells per ganglion about 4-fold higher when compared to GPEG (213.7 ± 50.4/PEG *vs* 53.3 ± 5.2 cells/GPEG, $P < 0.001$, Figure 1B) and a higher number of HuD-immunoreactive (HuD-IR) neurons per ganglion (+13.7%, Figure 1C). Frequency analysis in Figure 1F and Figure 1G describes differences between GPEG and PEG in terms of number of ganglia presenting 5 to 205 neurons. Moreover, PEG showed a different proportion of HuD-IR neurons and GFAP-immunoreactive (GFAP-IR) glial cells (+12.7%, $P < 0.05$), whereas GPEG presented a more homogenous distribution of both cell types. Notably, a higher number of neurons/ganglion (+12.7%, $P < 0.05$) and a lower number of glial cells/ganglion (-15.7%, $P < 0.05$) were detected in PEG compared to GPEG (Figure 1D).

Effect of LPS on the number of cells in pig and guinea pig enteric ganglia

GPEG exposed to increasing concentrations of LPS displayed a trend towards a decreased number of neurons/ganglion, which was statistically significant only at the concentration of 10 µg/ml (-22.3%, $P < 0.05$, Figure 2A). This effect was paralleled by an increased number of glial cells/ganglion (+22.2%, $P < 0.05$, Figure 2A). Conversely, no effect of LPS was detected on cell number in PEG cultures at any of the concentrations tested. Notably, the observed lower number of GFAP-IR glial cells compared with HuD-IR neurons was similar in all the experimental groups ($P < 0.05$, Figure 2B).

Characterization of pVW-MSCs phenotype

Flowcytometric analysis confirmed an unvaried immunophenotype of pVW-MSCs at the third passage after cryopreservation, displaying MSC profile. In line with the
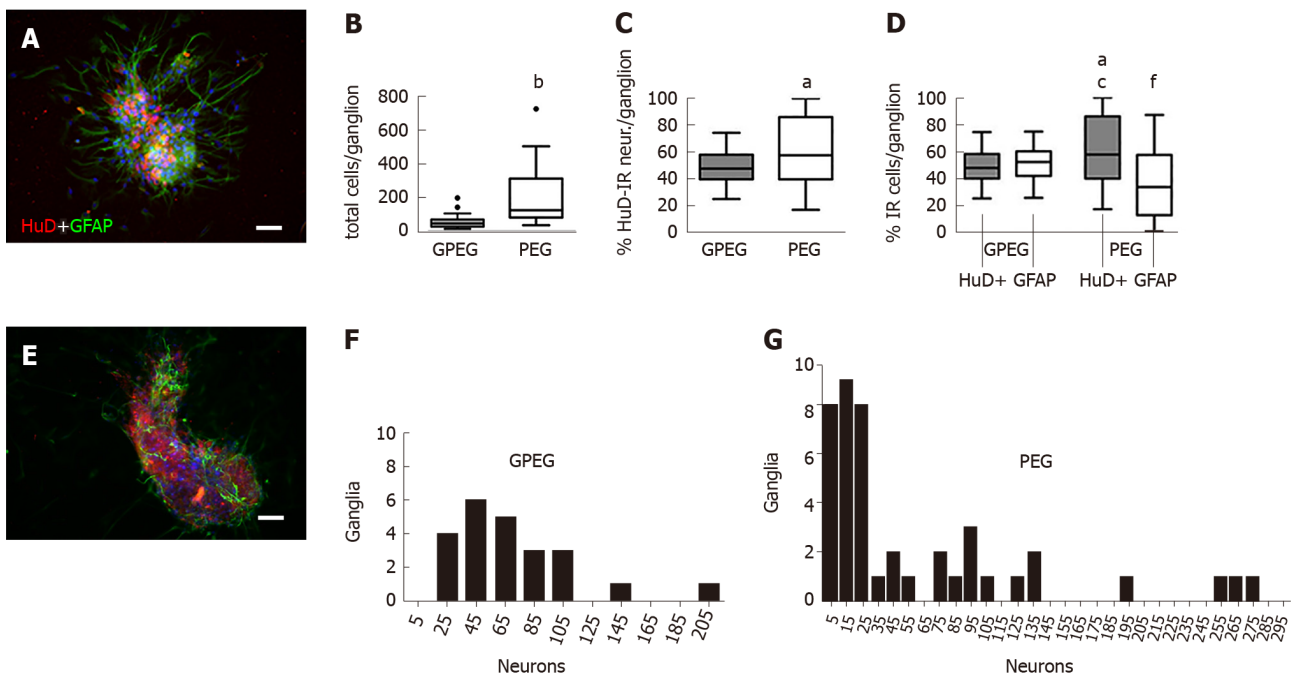
Figure 1 Descriptive analysis of the morphology and cellular composition of guinea pig and pig enteric ganglia after 2 d *in vitro*. A and E: Representative photomicrographs of guinea pig enteric ganglia (GPEG) and pig enteric ganglia (PEG) stained with HuD (red) and glial fibrillary acidic protein (GFAP) (green) antibodies directed to neurons and glial cells respectively (scale bar: 100 μm); B: Total number of cells per ganglion in GPEG, left gray box plot, and PEG, right white bars, cultures (53.3 ± 5.2 *vs* 213.7 ± 50.4 neurons per ganglion, [b]$P < 0.001$ *vs* GPEG); C: PEG showed a higher number of HuD-immunoreactive (HuD-IR) neurons compared to GPEG (+13.7%, [a]$P < 0.05$); D: PEG and GPEG comparison of HuD-IR neurons and GFAP-immunoreactive (GFAP-IR) glial cells: PEG presented a higher number of HuD-IR neurons compared to GFAP-IR glial cells (+28.4%, [a]$P < 0.05$). In comparison to GPEG, PEG showed a higher number of neurons (+12.7%, [c]$P < 0.05$) and a lower number of GFAP-IR glial cells (-15.7%, [f]$P < 0.01$); B-D: Values reported as Tukey box-plots were obtained by three independent experiments. F and G: Frequency analysis indicating the number of GPEG (F) and PEG (G) presenting 5 to 205 neurons. GPEG: Guinea pig enteric ganglia; PEG: Pig enteric ganglia; GFAP: Glial fibrillary acidic protein; GFAP-IR: Glial fibrillary acidic protein-immunoreactive; HuD-IR: HuD-immunoreactive.

criteria for MSC characterization[18] more than 96% of the cell population analyzed was positive for the markers of mesenchymal stemness, CD105, CD90, CD56, CD44, and less than 2.5% was positive for the hematopoietic markers CD45 and CD34 (Figure 3).

Effect of pVW-MSCs mediators on GPEG and PEG exposed to LPS

Thereafter, we tested the effect of medium conditioned by pVW-MSCs (CM) on GPEG and PEG cultured with LPS 1 μg/mL (LPS1). The concentration of 1 μg/mL was chosen in order to resemble a plausible pathophysiological condition of a high bacterial overload. Both guinea pig and pig cultures did not show any significant change in the number of HuD+ neurons after treatments (Figure 4A and B, white columns), whereas glial cell number varied significantly (Figure 4A and B, gray columns). In particular, GPEG cultures showed a higher number of glial cells as a result of co-treatment with CM+LPS1, compared to control and LPS1 groups (+13.9%, $P < 0.001$; +16.5%, $P < 0.01$, respectively). As for PEG cultures an increased number of GFAP+ glial cells was observed in CM group compared to control (+13.6%, $P < 0.05$) and LPS1 groups (+20.2%, $P < 0.05$). In addition, number of glial cells was higher in GPEG treated with CM+LPS1 compared to LPS1 (+14.2%, $P < 0.05$). The main interspecies difference was the variation of number of glial cells exposed to CM, which increased in PEG but not in GPEG cultures compared to the relative control (Figure 4A and B, third gray columns).

Morphometric analysis of ganglia upon treatment with pVW-MSC-conditioned medium

As most of the observed differences regarded glial rather than neuronal cells, we proceeded with a morphometric analysis of glial processes protruding outward the ganglion center area measuring the extent of the ganglion expanded area (Gang. Exp.%, Figure 5A). PEG morphology underwent more substantial changes in comparison to GPEG cultures which did not show any significant change following treatments showing a trend towards decreased Neur.Exp. (not statistically significant) after LPS1 treatment compared to control and CM groups. Furthermore, CM+LPS1 induced a marked increase of Gang.Exp. which was approximately 2 fold higher compared to both LPS1 and control groups (+43.2% *vs* CTRL, $P < 0.01$, Figure 5B).
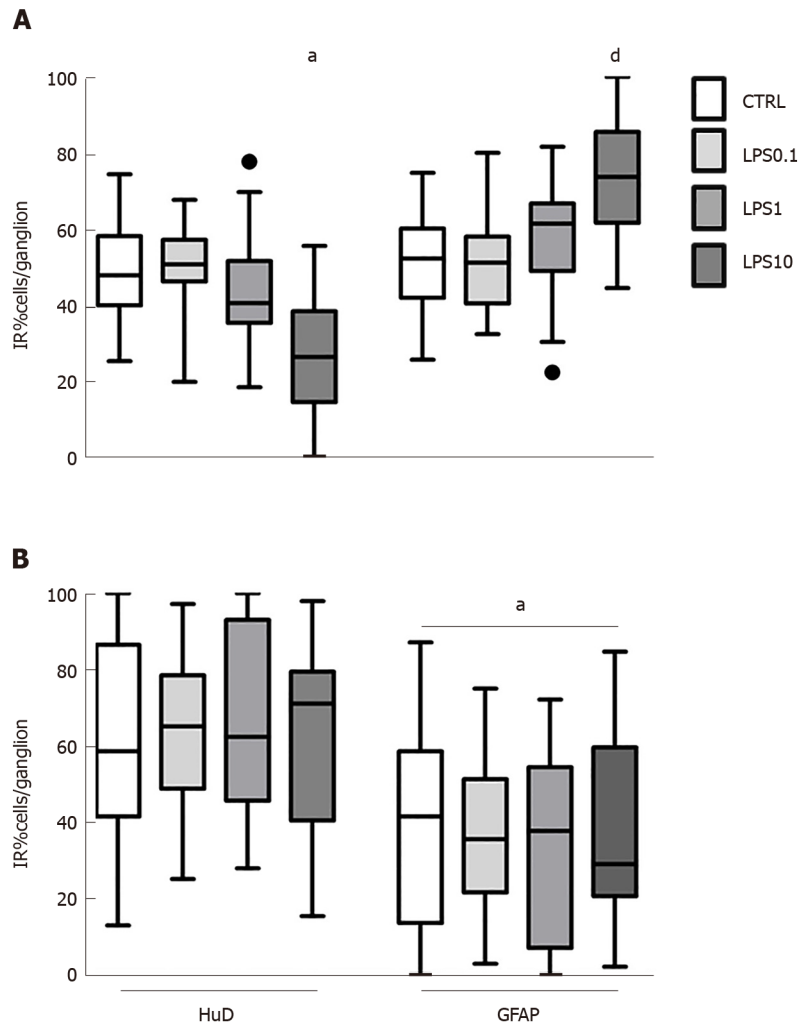
Figure 2 Effect of increasing concentration of lipopolysaccharide on enteric ganglia' HUD+ neurons and GFAP+ glial cells. A: In guinea pig-derived enteric ganglia - lipopolysaccharide (LPS) at 10 μg/mL decreased number of HuD-immunoreactive (HuD-IR) neurons (left columns) and increased proliferation of glial fibrillary acidic protein-immunoreactive (GFAP-IR) glial cells (right columns - HuD-IR neurons LPS10 *vs* CTRL, 22.3%, [a]*P* < 0.05; GFAP-IR glial cells LPS10 *vs* CTRL, +22.2%, [d]*P* < 0.01); B: Conversely, in pig enteric ganglia the number of glial cells at every LPS concentration tested did not change and was significantly lower compared to ganglionic neurons. [a]*P* < 0.05. LPS: lipopolysaccharide; GFAP-IR: Glial fibrillary acidic protein-immunoreactive; HuD-IR: HuD-immunoreactive.

DISCUSSION

The present study shows higher reactivity to MSC mediators of glial cells in pig compared to guinea pig myenteric ganglia. In particular, we tested the effect of CM derived by pVW-MSCs cultures on myenteric ganglia isolated from ileal tissue of GPEG and PEG. These primary cultures exposed to LPS combined with pVW-MSCs medium showed a more pronounced proliferation and differentiation in PEG compared to GPEG. This finding suggests a different and higher response of neuroimmune cells in higher mammals, which could impact on translational aspects of current research on cell-based therapies.

In the present study, we reported interspecies differences in the cellular composition of GPEG and PEG, with a higher neuronal/glial cells ratio in the latter, which is in line with previous findings[29]. Furthermore, we described a slight decrease in the number of neurons with a correspondent increase of glial cells as a result of increasing micromolar concentrations of LPS in GPEG, but not in PEG. Finally, we detected a marked modification of glial cell number and morphological modifications of PEG in response to CM derived by pVW-MSCs cultures.

The higher number of neurons detected in PEG is in line with previous findings describing an anatomical correlation in the size of myenteric ganglia and number of cells per ganglion in large mammals[30]. Moreover, PEG size and number of cells were more variable compared to GPEG, partially reflecting ganglia composition observed
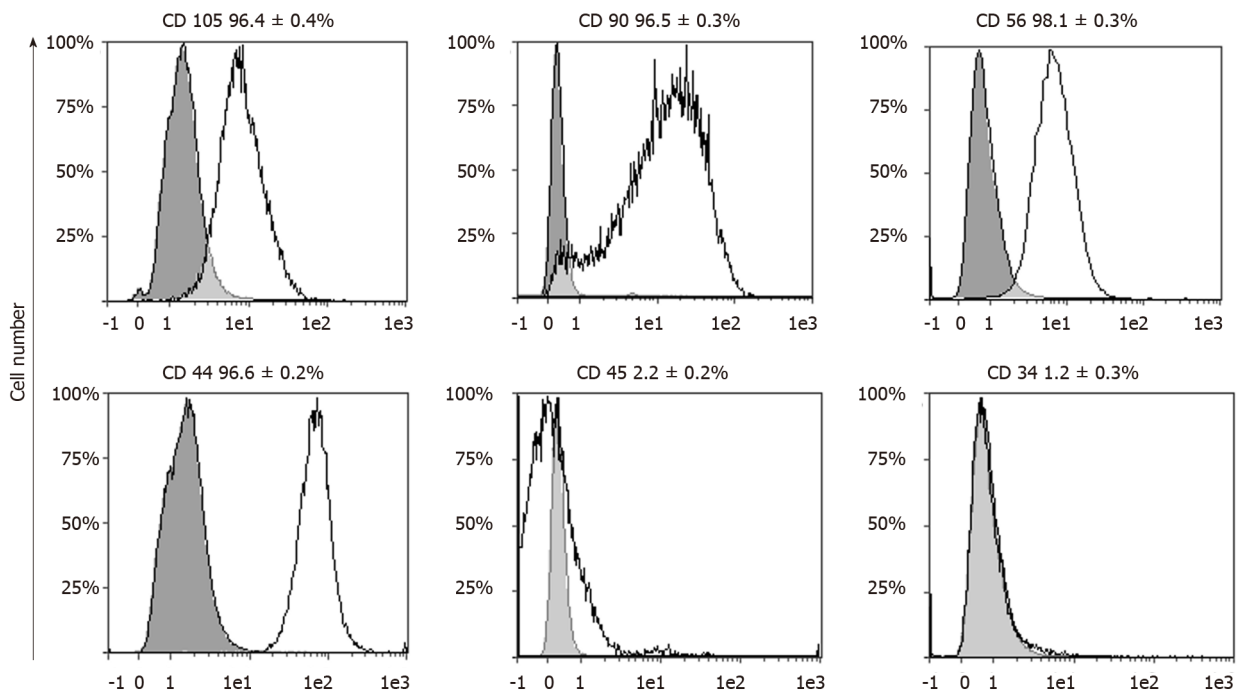
Figure 3 Flowcytometric analysis of cell-surface markers in porcine vascular wall mesenchymal stromal cells. Each graph shows the percentage of cells expressing the specific marker reported [white area under the curve (AUC)] and the relative negative control (gray AUC, cells not incubated with any antibodies). This analysis confirmed the mesenchymal stromal cell-like immune profile of porcine vascular wall mesenchymal stromal cells: CD105, CD90, CD56, CD44 were highly expressed (> 96%) while the hematopoietic markers CD45 and CD43 were nearly absent (< 2.5%). AUC: Area under the curve.

in larger mammals, including humans[31]. Our findings show a low glial cells/neurons ratio, particularly in PEG, which is in line with previous published data[29]. This disproportion is easily filled within 48 h of culture, due to the rapid proliferation of glial cells. In order to avoid a possible confounder, we chose a shorter time (24 h) to limit the proliferation of glial cells, so as to detect small variations in number and morphology of ganglia resulting after treatment.

Notably, our data of cell count analysis correspond to micromolar LPS concentrations as a result of previous tests performed with nanomolar concentrations. This analysis did not provide any measurable difference between groups (10-100 nM, data not shown). Moreover, the scarce decrease of cell number in GPEG and the absence of any effect in PEG cultures even with LPS at the highest concentrations (10 μM) reflects a remarkable resilience of myenteric neurons, already reported in previous works[32]. The slight decrease of neuronal cells at 10 μM of LPS in GPEG could be ascribed to a lower sensitivity of guinea pigs to LPS compared to pigs, which was tested in previous studies on LPS-induced endotoxic shock[33,34]. However, Schuster and colleagues described a counterintuitive effect of LPS promoting neuronal viability and stemness in myenteric ganglia derived by MP of newborn mice[32]. Differently from this work, our data did not show a higher neuron number as a result of LPS treatment. Rather, most of the variations observed, as probably due to age-related features of the animals used (young animals rather than newborns), regarded glial cell number, which markedly varied upon treatment with pVW-MSCs supernatants, while it did not evoke any measurable change on the neuronal component in either pig or guinea pig cultures. Indeed, CM derived by pVW-MSCs alone or combined with 1 μg/mL LPS induced a higher number of glial cells in PEG, while in GPEG-treated samples an akin effect was found only after the co-treatment, suggesting a synergic activity of pVW-MSC-secreted molecules and LPS in promoting glial cells mitosis in both models. This observation is in accordance with the properties showed by brain vascular pericytes which favor glial cells' phenotype, being also spatially in close relation with this cell type in brain vessels[35,36]. Indeed, pVW-MSCs, along with a MSC-like immune profile, exhibited an intrinsic pro-angiogenic features in previous studies[19,27]. In addition, both LPS and MSCs promote the activation of glial cells in brain-derived ganglia. In particular, a recent study *in vitro* described the induction of glia proliferation induced by Wharton-jelly-derived MSCs[37], while *in vivo* injection of LPS induced an overexpression of the glial marker GFAP in brain tissue[38]. Interestingly, we observed a substantial variation of this cell population in swine but not in guinea pig primary cultures. Allegedly, this might be
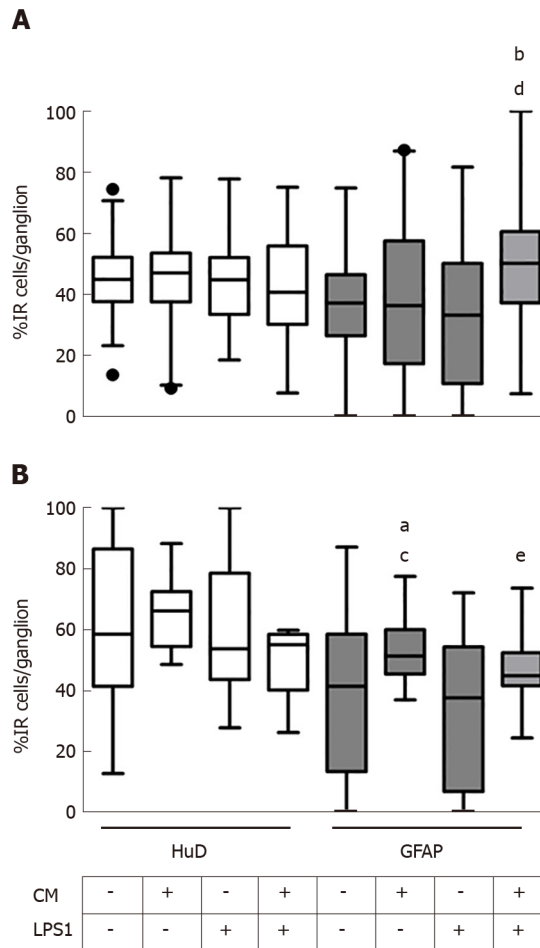
Figure 4 Effect of porcine vascular wall mesenchymal stromal cells supernatants on the number of neurons (left white bars) and glial cells (gray right bars) exposed to lipopolysaccharide 1 μg/mL. A: The number of neurons in guinea pig enteric ganglia did not change significantly upon any of the treatment tested, whereas co-treatment with conditioned medium (CM) and lipopolysaccharide 1 μg/mL (LPS1) (CM+LPS1) increased the number of glial cells compared to control (CTRL) and LPS1-treated ganglia (+13.9%, $^bP < 0.001$; +16.5%, $^dP < 0.01$, respectively); B: The number of neurons in pig enteric ganglia did not change as a result of any of the treatment tested. Conversely glial cell number was higher in the CM group compared to control (+13.6%, $^aP < 0.05$) and LPS1 (+20.2%, $^cP < 0.05$). CM+LPS1 co-treatment increased the number of glial cells compared to LPS1-treated ganglia (+14.2, $^eP < 0.05$). LPS1: Lipopolysaccharide 1 μg/mL; CM: Conditioned medium. GFAP: Glial fibrillary acidic protein.

due to the species correspondence of porcine enteric glia with pVW-MSCs, which would reflect the phylogenetic differences previously reported in signaling modalities for MSC immunomodulation[39]. Indeed, in humans, non-human primates and pigs, immunomodulation is a mechanism dependent by indoleamine 2,3-dioxygenase secretion whereas in rodents the same mechanism is associated with inducible nitric oxide synthase expression and nitric oxide production[21,39]. Whether the observed increase in number and shape of glial cells should be associated with a compensative/therapeutic rather than a noxious *stimulus* should be addressed by further investigations on cytokine expression patterns. In this sense, an exhaustive characterization of molecular mechanisms activated by MSC-derived bioactive molecules was beyond the scope of our analysis.

Taken together, these lines of evidence suggest an effect of pVW-MSCs mediators on glial cells promoting neuronal remodeling and confirm the paramount role of this cell type in modulating immune-mediated changes of the ENS. A further characterization of the type of glial cells involved in these changes is warranted. Moreover, the observed interspecies differences should be taken into consideration in future investigations of immune-mediated response to MSCs secretome in rodents models.
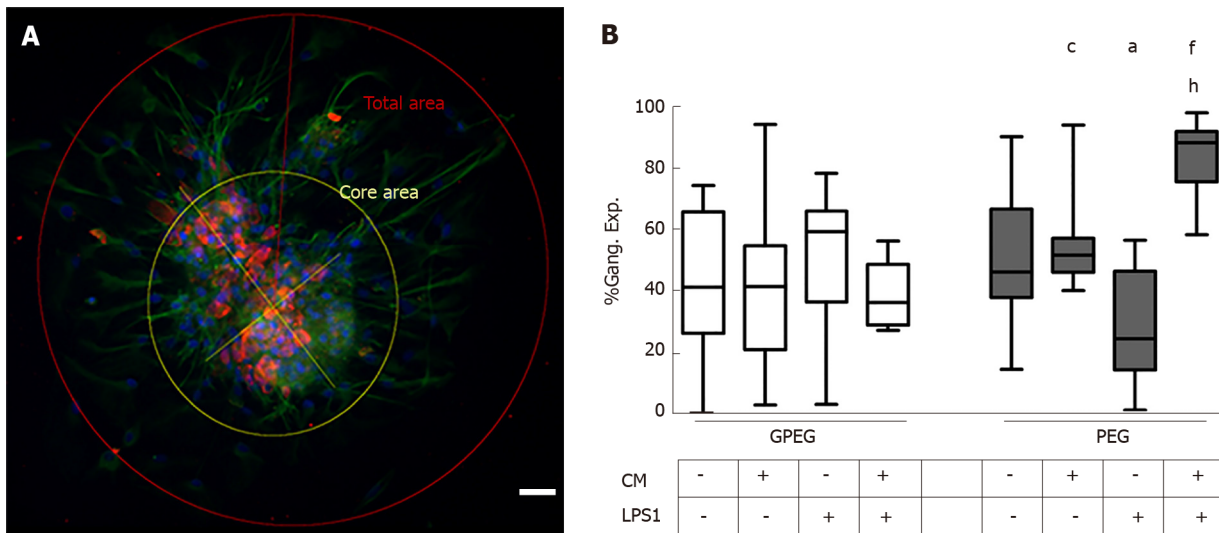
Figure 5 Effect of porcine vascular wall mesenchymal stromal cells supernatants on ganglion expansion. A: Representative photo of the morphometric analysis performed to compare glial cells processes elongating from ganglion's cores under different experimental conditions (scale bar: 100 μm); B: Relative area occupied by glial processes of guinea pig (left, gray bars) and pig enteric ganglia (right white bars). Guinea pig enteric ganglia did not show any significant difference between the different treatment groups. Conversely, pig enteric ganglia were more subjected to morphological changes: There was a decrease of the expanded area in the group treated with lipopolysaccharide 1 μg/mL (LPS1) compared to control (-42.88%, [a]$P < 0.05$). Moreover, conditioned medium (CM) derived by porcine vascular wall mesenchymal stromal cells evoked a higher protrusion of glial processes than LPS1 alone (+36.8%, [c]$P < 0.05$) which was remarkably higher in combination with LPS (CM+LPS1, +43.2% *vs* CTRL, [f]$P < 0.01$; +60.9% *vs* LPS1, [h]$P < 0.01$). LPS1: Lipopolysaccharide 1 μg/mL; CM: Conditioned medium; GPEG: Guinea pig enteric ganglia; PEG: Pig enteric ganglia.

ARTICLE HIGHLIGHTS

Research background

There is growing interest on mesenchymal stromal cells (MSC) as a novel therapeutic strategy to treat auto-immune and inflammatory diseases. However, identifying optimal MSC sources and limited reliability of current experimental models still represent a challenge in this field. Pigs represent more closely human physiology and an accessible resource for *ex vivo* procedures. Recently, our group isolated a population of pericytes from porcine aortic wall with an MSC profile, currently cited as porcine vascular wall-MSC (pVW-MSC).

Research motivation

Inflammatory bowel diseases (IBDs), comprising the two major forms ulcerative colitis and Crohn's disease, are characterized by an aberrant immune response leading to severe damage of the intestinal wall and functioning. Current trials are evaluating the application of cell-based therapies for the treatment of IBDs. The present study describes the effect of pVW-MSC-conditioned medium (CM) on enteric ganglia in two *ex vivo* models of IBDs in order to investigate a potential development of MSC-based treatment of IBDs.

Research objective

To evaluate the effect of pVW-MSC secretome on survival and differentiation of enteric ganglionic cells isolated by guinea pigs (GPEG) and pigs (PEG) and exposed to lipopolysaccharide (LPS).

Research methods

The expression of standard MSC markers in pVW-MSC were assessed by flow cytometry. Increasing concentration of LPS were tested in both GPEG and PEG cultures. CM derived by pVW-MSC cultures were added alone or in combination with 1μg of LPS in GPEG and PEG cultures. Ganglionic cells were double-stained with antibodies directed to the pan-neuronal marker, HuD and the glial fibrillary acidic protein, GFAP. Cell count and morphometric analysis were performed to determine changes of neuronal and glial population.

Research results

Guinea-pig neurons and glial cells decreased and increased respectively in response to high concentrations of LPS. These changes were not observed in pig primary cultures. pVW-MSC secretome increased the number and differentiation of glial cells compared to neurons with a more pronounced effect in PEG and in combination with LPS.

Research conclusions

These data showed a higher resilience of pig enteric ganglia to the main bacterial product LPS compared to guinea pig and a higher responsiveness of glial cells to pVW-MSC secreted mediators.

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