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Sertoli Cells Possess Immunomodulatory Properties and the Ability of Mitochondrial Transfer Similar to Mesenchymal Stromal Cells

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Abstract

It is becoming increasingly evident that selecting an optimal source of mesenchymal stromal cells (MSCs) is crucial for the successful outcome of MSC-based therapies. During the search for cells with potent regenerative properties, Sertoli cells (SCs) have been proven to modulate immune response in both in vitro and in vivo models. Based on morphological properties and expression of surface markers, it has been suggested that SCs could be a kind of MSCs, however, this hypothesis has not been fully confirmed. Therefore, we compared several parameters of MSCs and SCs, with the aim to evaluate the therapeutic potential of SCs in regenerative medicine. We showed that SCs successfully underwent osteogenic, chondrogenic and adipogenic differentiation and determined the expression profile of canonical MSC markers on the SC surface. Besides, SCs rescued T helper (Th) cells from undergoing apoptosis, promoted the anti-inflammatory phenotype of these cells, but did not regulate Th cell proliferation. MSCs impaired the Th17-mediated response; on the other hand, SCs suppressed the inflammatory polarisation in general. SCs induced M2 macrophage polarisation more effectively than MSCs. For the first time, we demonstrated here the ability of SCs to transfer mitochondria to immune cells. Our results indicate that SCs are a type of MSCs and modulate the reactivity of the immune system. Therefore, we suggest that SCs are promising candidates for application in regenerative medicine due to their anti-inflammatory and protective effects, especially in the therapies for diseases associated with testicular tissue inflammation.

Keyword Mesenchymal stem cells. Sertoli cells. Immunomodulation. Mitochondrial transfer

Introduction

Stem cells are recently intensively investigated for the therapy of various diseases. Results obtained from many clinical studies proved the safety of the application of mesenchymal stromal cells (MSCs) isolated from various tissues, but clinical outcomes of MSC transplantation still do not meet expectations [1]. Therefore, new approaches are being investigated, and the search for stem cells with better regenerative and immunomodulatory capacities continues. In this respect, the tissue source for MSCs isolation is crucial since MSCs from different sites of the body do not possess the same properties [2], which influences the therapy outcome [3, 4]. Recently, the International Society for Cell & Gene Therapy (ISCT®) defined mesenchymal stem cells are a minor population of progenitor cells with differentiation and self-renewal ability within a larger bulk population of mesenchymal stromal cells with known immunomodulatory, homing and secretory funcitons [5]. Hence here, in this article we will refer to MSCs as the mesenchymal stromal cells. Regarding the ongoing search for suitable cell type for therapies requiring immune modulation, Sertoli cells (SCs) can be studied as promising candidates.

In the specific environment of testes, SCs have been described to possess a similar biological function as MSCs. Adult SCs have been previously considered terminally differentiated cells with the only function to protect and nourish spermatogonial stem cells. However, this opinion has been

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challenged, as it was proved that adult SCs could regain their proliferation potential after transplantation [6]. According to the current knowledge, the primary function of SCs is, in addition to nourishing and supporting germ cells, to protect them from immune destruction, form blood-testis barrier and provide immune privilege. Besides, it has been documented that SCs provide support and a tolerogenic environment for co-transplanted cells even across immunological barriers in various in vivo models [7].

Recently, it has been proposed that SCs could be a kind of MSCs. Chikhovskaya et al. [8] demonstrated that somatic testicular cell cultures form colonies resembling MSCs. SCs also possess a phenotype similar to MSCs, including the ability to undergo differentiation along mesodermal lineages [9] and the expression of MSC-like surface markers [10]. These studies suggested that SCs could be a stem cell population, but this hypothesis has not been confirmed.

An essential property of MSCs is the ability to modulate immune response [11]. Significant immunosuppressive properties of SCs and their ability to promote cell growth have also been described in this regard [12], but mechanisms of the suppressive effect of SCs remain unclear. Recent studies have shown that the suppressive ability of SCs, similarly to MSCs, depends on the used model (animals, disease, and experimental design). SCs modulate the reactivity of the innate immune system, including the induction of an alternative M2 phenotype of macrophages and suppression of the co-stimulatory abilities of dendritic cells [13]. The effect of SCs on T-cell responses, particularly the shift towards T helper type 2 (Th2) and regulatory T cell (Treg) type of immune response, has been demonstrated, as documented by the upregulation of IL-10 and TGFβ production, as well as the increased Treg number after co-cultivation with naïve T cells [14]. Expression of molecules participating in the tolerance, including PD-L1, FasL and indoleamine 2,3-dioxygenase, also plays an essential role in SC-mediated immunomodulation [15]. Furthermore, SCs have been a potent immunoregulatory tool in in vivo models of diabetes, neurodegenerative diseases and transplantation [9, 16–18].

The mitochondrial transfer has been described as one of the mechanisms, which MSCs use to support anti-inflammatory conditions and cell survival [19]. This mechanism has been identified as a critical pathway for the Th17 to Treg switch [20]. In various models, MSCs transferred mitochondria to cardiomyocytes, bronchial epithelial cells or cortical neurons [21–23]. The mitochondrial transfer has never been described in SCs; however, connexin43, which is one of the key proteins in this process, is a major protein in forming tight junctions and blood-testis barriers by SCs [24].

The ability of SCs to promote cell growth, their beneficial anti-inflammatory effects and the protection of co-transplanted tissue, together with the fact that SCs can be easily isolated from patient testicular biopsies performed routinely by fertility clinics, made them promising candidates in the field of tissue repair and regeneration. Besides, due to the non-immunogenic properties of SCs, allogeneic cells isolated from a donor with healthy SCs can be used. Several groups reported that transplantation of MSCs or their products could restore spermatogenesis and fertility in various models [25–27]. Confirmation of SC stem-like properties and further elucidation of molecular aspects of immunomodulation, differentiation and mechanisms of their action may enable new approaches for their application with the aim to support the regeneration of testicular damage. Therefore, the objective of this study was to verify the stem properties of SCs and compare them with those of MSCs, including their immunomodulatory potential. We also examined the ability of mitochondrial transfer as one of the mechanisms by which MSCs and SCs could provide protection of tissue from acute damage.

Materials and Methods

Animals

Female BALB/c mice (spleen cell isolation) at the age of 8–12 weeks and male BALB/c mice (SC isolation) at the age of 3 weeks were obtained from the breeding unit of the Institute of Molecular Genetics of the Czech Academy of Sciences, Prague, Czech Republic. The present study was approved by the Animal Ethics Committee of Charles University, and all experimental procedures were performed following the guidelines for the care and use of laboratory animals.

Isolation of Adipose-Derived MSCs

Adipose-derived MSCs were isolated from inguinal fat pads of BALB/c mice as we have described [28], cultured in Dulbecco's modified Eagle medium (DMEM, PAA Laboratories, Pasching, Austria) supplemented with 10% FBS (Sigma-Aldrich Corporation, St. Louis, MO, USA), antibiotics (100 mg/ml of streptomycin, 100 U/ml of penicillin) and 10 mM Hepes buffer, and maintained in culture as adherent monolayers. Cells between passages 3 and 5 were used in the experiments.

Isolation of Sertoli Cells

Briefly, testes from 3-5 weeks old male mice were decapsulated with tweezers and digested with Collagenase II and DNase I in PBS, 20 min in shaking bath (32 °C), centrifuged (10 min, 800 g) and filtered through 70 μm and then through 40 μm cell strainer. Cells on the 40 μm cell strainer were washed out by centrifugation (10 min, 800 g). The cell

suspension was plated on DSA (lectin from Datura Stramonium, Sigma-Aldrich) coated flask, washed 1 h after plating with warm DMEM medium and cultured in a complete DMEM medium supplemented with glutamine, LIF (0.1 ng/ml, Peprotech Rocky Hill, NJ, USA) and FSH (0.5 ng/ml, Sigma-Aldrich) for 3 weeks with regular exchange of medium, then passaged twice a week. Cells were maintained in culture as adherent monolayers, and between passages 3–6 were used in the experiments.

Characterization of Surface Markers by Flow Cytometry

SCs and MSCs were harvested between passages 3–5 and washed with PBS/0.5% BSA and incubated for 30 min on ice with FITC-labeled monoclonal antibody (mAb) anti-CD90.2 (clone 30-H12, SONY), PE-labeled mAb anti-CD105 (clone MJ7/18, BioLegend San Diego, CA, USA), PE-labeled mAb anti-CD73 (clone eBioTY/11.8, eBioscience, San Diego, CA, USA), FITC-labeled mAb anti-CD44 (clone IM7, BioLegend), FITC-labeled mAb anti-CD45 (clone 30-F11, BioLegend), FITC-labeled mAb anti-CD11b (clone M1/70, BioLgenend), PE-labeled mAb anti-CD34 (clone HM34, BioLegend), PE-labeled mAb anti-CD31 (clone 390, BioLegend). Unstained cells were used as a control. A total of 40 000 cells were analyzed after the exclusion of dead cells and debris.

Macrophages were prepared by washing the peritoneal cavity of unstimulated BALB/c mice as we described elsewhere [29], and cells (5×10^5 cells/ml) were plated in 24-well tissue culture plates (Nunc, Roskilde, Denmark) in a volume of 1 ml of RPMI-1640 medium supplemented with 10% FBS (Sigma-Aldrich), antibiotics (100 mg/ml of streptomycin, 100 U/ml of penicillin) and 10 mM Hepes buffer (referred as complete RPMI-1640 medium), for 48 h in the presence or absence of SCs/MSCs (peritoneal cells: SC/MCs at a ratio 1:5, 1:10 or 1:20). To determine the phenotype of macrophages, cells were harvested, washed with PBS/0.5%BSA and incubated for 30 min on ice with Alexa Fluor 700-labeled mAb anti-CD45 (clone 30-F11, BioLegend), PE-labeled mAb anti-F4//80 (clone BM8, BioLegend), FITC-labeled aAb anti-CD206 (clone C068c2, BioLegend). Macrophages incubated alone were used as a control. A total of 50 000 cells were analyzed after exclusion of dead cells and debris. Representative flow cytometry dot plots illustrating the gating strategy are shown in Supplementary Fig. S1. Five independent experiments were performed for each part of the study.

In all experiments, dead cells were stained with Hoechst 33,258 fluorescent dye (Sigma-Aldrich). Data were collected using LSR II cytometer (BD Bioscience Franklin Lakes, NJ, USA) and analyzed using GateLogic 400.2A software (Invai, Mentone, Australia).

RT-PCR

Total RNA was isolated from cultured SCs and murine testicular cell suspension using E.Z.N.A.® Total RNA Kit I (Omega Bio-Tek, Georgia, GA, USA) according to manufacturer's instructions, including in-column DNase treatment. Reverse transcription was performed by the SuperScript™ Reverse Transcriptase (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's instructions. Primer sequences are listed in Table S1.

Osteogenic, Chondrogenic and Adipogenic Differentiation of SCs and MSCs

SCs or MSCs were cultivated in DMEM medium to ensure mid-log growth phase confluence (60–80%). Then cells were gently harvested, and depending on the type of differentiation, were seeded into multi-well plates or Petri dishes. Cells underwent osteogenic differentiation using StemPro® Osteogenesis Differentiation Kit (Thermo Fisher Scientific), according to the manufacturer's instructions. Osteocytes were stained with 2% Alizarin Red S solution (Sigma-Aldrich) for 15 min. A micromass culture was generated from SCs or MSCs and cultured in media prepared from StemPro® Chondrogenesis Differentiation Kit (Thermo Fisher Scientific) to induce chondrogenic differentiation. Droplets with a volume of 5 µl of the cell suspension were seeded in the centre of the 6-well plate to generate the micromass. After incubation for 2 h under high humidity conditions, chondrogenesis media was added to the cultures. Chondrocytes were detected using 1% Alcian Blue solution on day 21. Adipogenesis was induced using StemPro® Adipogenesis Differentiation Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Expanded SCs or MSCs were seeded into culture flasks and cultured in an adipogenesis differentiation medium. Lipid droplets were detected on day eight by Oil Red O (Sigma-Aldrich) staining. All samples were evaluated under a light microscope.

Detection of Apoptosis

Spleen cells (1×10^6 cells/ml) were cultured in a volume of 1 ml complete RPMI 1640 medium in 24-well tissue culture plates stimulated with Concanavalin A (ConA, 1.25 µg/ml, Sigma-Aldrich) for 48 h in the presence or absence of SCs or MSCs (SCs/MSCs: spleen cells ratio 1:10 or 1:20). Cells were harvested, washed with PBS/0.5% BSA and incubated for 30 min on ice with Alexa Fluor 700-labeled mAb anti-CD45 (clone 30-F11, BioLegend), FITC-labeled mAb anti-CD4 (clone GK1.5, BD Pharmingen, San Jose, CA, USA). After washing with PBS/0.5% BSA, cells were stained for Annexin V using Annexin V detection kit according to the manufacturer's protocol (Apronex, Jesenice, Czech

Republic). Dead cells were excluded using Hoechst 33258 (Sigma-Aldrich), added 15 min before flow cytometry analysis. Data were collected using LSR II cytometer (BD Bioscience) and analyzed using GateLogic 400.2A software (Invai). Representative dot plots illustrating the gating strategy are shown in Supplementary Fig. S2, gated as Ki67. A total of five independent experiments were performed.

Intracellular Detection of Transcription Factors

Spleen cells (1×10^6 cells/ml) were cultured in a volume of 1 ml of complete RPMI 1640 medium in 24-well tissue culture plates stimulated with ConA, (1.25 µg/ml) for 72 h in the presence or absence of SCs or MSCs (SCs/MSCs:Spleen cells ratio was 1:10 or 1:20). Cells were harvested, washed with PBS/0.5% BSA and incubated for 30 min on ice with Alexa Fluor 700-labeled mAb anti-CD45 (clone 30-F11, BioLegend), FITC-labeled mAb anti-CD4 (clone GK1.5, BD Pharmingen) and Live/Dead Fixable Violet Dead Cell Stain Kit (Thermo Fisher Scientific) for staining of dead cells. Cells were then fixed and permeabilized using a Foxp3 Staining Buffer Set (eBioscience) according to manufacturer's instructions, before staining for 30 min with APC-labeled mAb anti-Foxp3 (clone FJK-16 s, eBioscience), PE-labelled mAb anti-RORγt (clone AFKJS-9, eBioscience) or PE-labeled mAb anti-Ki67 (clone 16A8, BioLegend). A total of 40 000 cells were analyzed after exclusion of dead cells and debris. Data were collected using LSR II cytometerBD Bioscience) and analyzed using GateLogic 400.2A software (Invai). Gating strategy is shown in Supplementary Fig. S2. A total of five independent experiments were performed.

Intracellular Detection of Cytokines

Spleen cells (1×10^6 cells/ml) were cultured in a volume of 1 ml of complete RPMI 1640 medium in 24-well tissue culture plates stimulated with ConA (1.25 µg/ml) for 48 h in the presence or absence of SCs or MSCs (SCs/MSCs:spleen cells ratio 1:10 or 1:20). To analyze intracellular cytokine production, Phorbol 12-Myristate 13-Acetate (PMA, 20 ng/ml, Sigma-Aldrich), Ionomycin (500 ng/ml, Sigma-Aldrich), Brefeldin A (5 µg/ml, eBioscience) were added to the cultures for at least 4.5 h of the 48 h incubation period. Cells were harvested, washed with PBS/0.5% BSA and incubated for 30 min on ice with Alexa Fluor 700-labeled mAb anti-CD45 (clone 30-F11, BioLegend), FITC-labeled mAb anti-CD4 (clone GK1.5, BD Pharmingen) and Live/Dead Fixable Violet Dead Cell Stain Kit (Thermo Fisher Scientific) for staining of dead cells. Cells were then fixed and permeabilized using a Fixation and Permeabilization Kit (eBioscience) according to the manufacturer's instructions. The cells were intracellularly stained for 30 min with PE-labeled mAb anti-TNFα (clone MP6-XT22, eBiosciece), APC-labeled mAb anti-IL-2 (clone JES6-5H4, eBioscience), APC-labeled mAb anti-IL-17A (clone eBio17B7, eBioscience). A total of 40 000 cells were analyzed after exclusion of dead cells and debris. Data were collected using LSR II cytometer (BD Bioscience) and analyzed using GateLogic 400.2A software (Invai). Representative dot plots illustrating the gating strategy are shown in Supplementary Fig. S3. A total of five independent experiments were performed.

Mitochondrial Transfer from SCs and MSCs to Immune Cells

Spleen cells (1×10^6 per well) were cultured in a volume of 1 ml of complete RPMI 1640 medium in 24-well tissue culture plates. SCs and MSCs were stained for mitochondria using MitoTracker® Red CMXRos (MiTT, Thermo Fisher Scientific), according to the manufacturer's instruction at the concentration 100 nM for 30 min. Stained SCs or MSCs were added to spleen cells in the ratio 1:20 (SCs/MSCs: spleen cells) and cultured together for 3 h. Cells were harvested after co-cultivation, washed with PBS/0.5% BSA and incubated for 30 min on ice with Alexa Fluor 700-labeled mAb anti-CD45 (clone 30-F11, BioLegend), and Hoechst 33258 was used for dead cells staining and exclusion. Mitochondrial transfer from SCs or MSCs to immune cells was determined as a MiTT positive population gated on $CD45^+$ to exclude SCs and MSCs. A total number of 60 000 cells were analyzed after exclusion of dead cells and debris. Data were collected using LSR II cytometer (BD Bioscience) and analyzed using GateLogic 400.2A software (Invai).

Immunostaining of Co-cultures of Spleen Cells with SCs or MSCs – Mitochondrial Transfer Visualization

Spleen cells (1×10^5 per well) were cultured in a volume of 2 ml of complete DMEM medium in 29 mm Glass bottom dishes (Cellvis, Sunnyvale, California, USA) together with SCs and MSCs stained for mitochondria using MiTT, in ratio 1:20 (SCs/MSCs: spleen cells) for 3 h. Cell suspensions were washed with PBS and fixed with 4% paraformaldehyde in PBS for 15 min, permeabilized by 0.2% Triton x-100 in PBS, blocked by 1% BSA in PBS and stained for 1 h with rat anti-mouse CD45 primary antibody (1:200, BioLegend) and Phalloidin Green (Sigma-Aldrich) and goat anti-rat IgG (H+L) secondary antibody Alexa Fluor 647 (Thermo Fisher Scientific) for 2 h. Staining for nuclei and sample mounting was performed by Mowiol/DAPI (Sigma-Aldrich) and observed with LeicaDmi8 fluorescence microscope.

Statistical Analysis

For the statistical analysis, the program The Prism (GraphPad Software, San Diego, CA, USA) was used. The results are expressed as the mean ± standard error (SE). The statistical significance of differences between individual groups was calculated using one-way analysis of variance (ANOVA) and Tukey post hoc test. P values less than 0.05 were considered statistically significant.

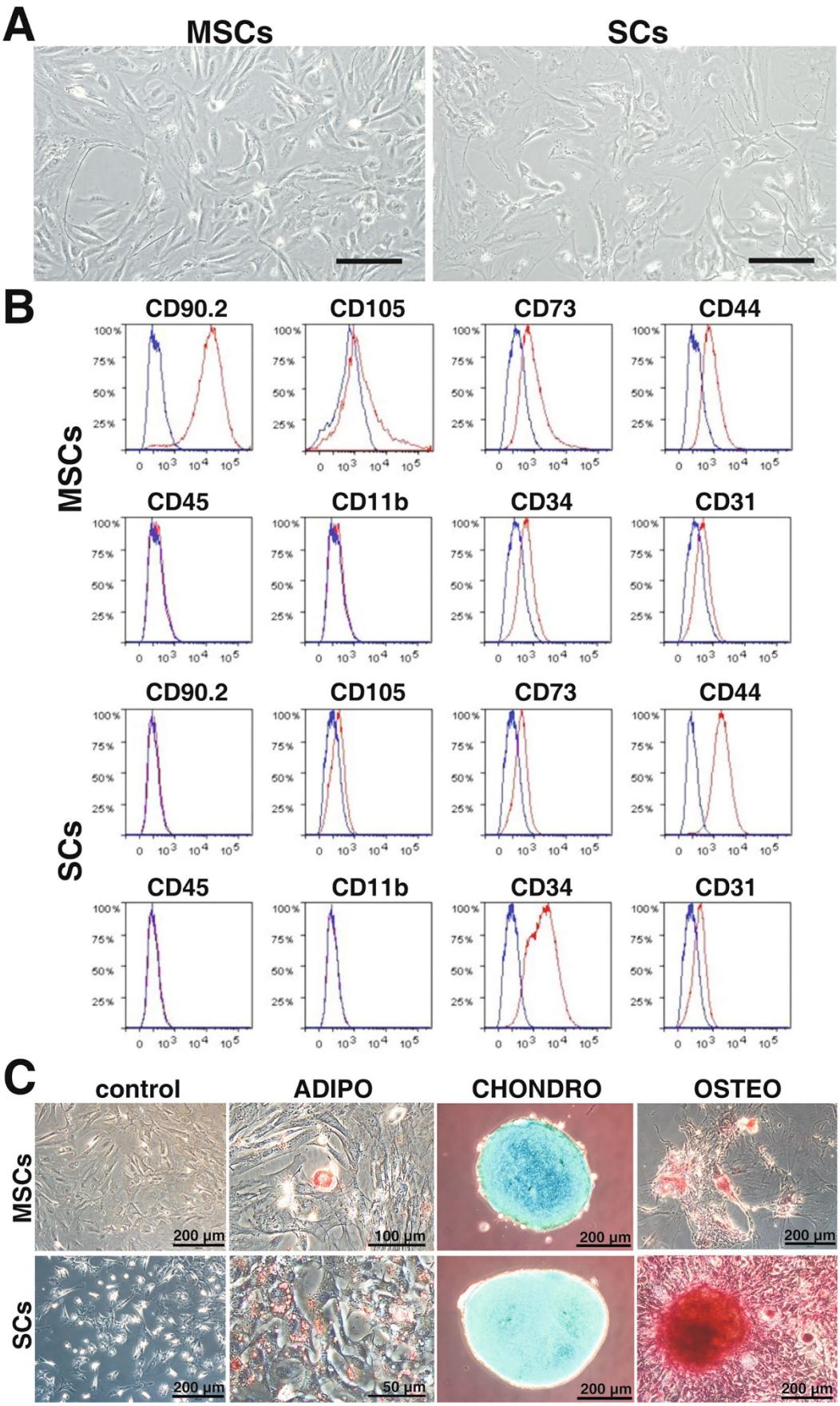
Results

SCs Fulfill Criteria of MSCs

We confirmed the characteristics of SCs by the expression of genes for Vimentin, CD44 (MSCs surface markers), Acta2 (Actin alpha2, testis associated marker) and Sox9 (transcription factor, SCs marker). SCs were also negative for germ cell markers Dazl, Ddx4, Ddx25 and Leydig cell markers

M. musculus gene symbol	Gene name	Gene transcript	Gene expression	
			testes	SC primoculture
Germ cell markers				
Dazl	deleted in azoodspermia-like	ENSMUST00000010736.7		
Ddx4	DEAD (Asp-Glu-Ala-Asp) box polypeptide 4	ENSMUST00000099166.9		
Ddx25	DEAD (Asp-Glu-Ala-Asp) box polypeptide 25	ENSMUST00000034612.6		
Mesenchymal stem cell surface markers				
CD44	CD44 antigen	ENSMUST00000005218.14		
Itgb1	integrin beta 1 (fibronectin receptor beta)	ENSMUST00000090006.11		
Thy1	thymus cell antigen 1, theta	ENSMUST00000114840.1		
Vim	vimentin	ENSMUST00000028062.7		
Leydig cell markers				
Cyp11a1	cytochrome P450, family 11, subfamily a, polypeptide 1	ENSMUST00000034874.13		
Cyp17a1	cytochrome P450, family 17, subfamily a, polypeptide 1	ENSMUST00000026012.7		
Testis associated markers				
Acta2	actin, alpha 2, smooth muscle, aorta	ENSMUST00000039631.8		
Lif	leukemia inhibitory factor	ENSMUST00000066283.11		
Sox9	SRY (sex determining region Y)-box 9	ENSMUST00000000579.2		

Fig. 1 Expression of genes characteristic for individual testicular cell populations. SCs did not express markers of germ cells and Leydig cells, but they expressed Sox9 (a marker of SCs), CD44, Vimentin (markers of MSCs) and Acta2 (actin alpha 2, testis associated marker). Data from one of two similar experiments are shown


A
MSCs
SCs
B
CD90.2
CD105
CD73
CD44
MSCs
CD45
CD11b
CD34
CD31
CD90.2
CD105
CD73
CD44
SCs
CD45
CD11b
CD34
CD31
100%
75%
50%
25%
0
10^3
10^4
10^5
C
control
ADIPO
CHONDRO
OSTEO
MSCs
SCs
200 µm
100 µm
50 µm

◂**Fig. 2** Characterization of MSCs and SCs. Adherence to the plastic surface is documented in the representative phase-contrast light microscopy image. Both cell populations isolated from the one young (4 weeks) male mouse **A**. The expression of cell surface markers on MSCs and SCs was determined by flow cytometry **B** MSCs and SCs were cultured in adipogenic, chondrogenic or osteogenic medium and stained with Oil Red O, Alcian Blue or Alizarin Red S, respectively. Representative images of differentiated MSCs and SCs are shown **C**

Cyp11a1, Cyp17a1 (Fig. 1). SCs were adherent to plastic (Fig. 2A), positive for CD44 and slightly positive for CD105 and CD73 used as markers defining MSCs [30], but were negative for CD90.2. Both cell populations were negative for CD45, CD11b. SCs were positive for the CD34 marker, whereas MSCs were slightly positive (Fig. 2B). Both MSCs and SCs were capable of differentiating into adipocytes, osteocytes and chondrocytes (Fig. 2C).

SCs Modulate CD4$^+$ T Cell Proliferation, Apoptosis and Phenotype

The anti-inflammatory effect of MSCs on T cells is well described [31]. Therefore, we measured several parameters, including proliferation, apoptosis and Treg/Th17 ratio and compared the effects of SCs with those of MSCs. MSCs suppress the proliferation of CD4$^+$ cells induced by ConA. Expression of a nuclear protein Ki67 was downregulated on CD4$^+$ cells after co-culture with MSCs; the suppression by SCs was less pronounced (Fig. 3A). SCs protected activated CD4 + cells from apoptosis, revealed by the presence of phosphatidylserine on the cell surface using Annexin V similarly to MSCs (Fig. 3B). As shown in Fig. 3C, SCs promoted CD4$^+$ T cell phenotype switch to anti-inflammatory. Treg/Th 17 ratio showed only a tendency to increase in the presence of MSCs in the culture, while in the presence of SCs, this ratio increased significantly.

SCs Suppress the Production of Inflammatory Cytokines by Activated CD4$^+$ T Cells

To further determine the activation of T cells cultivated in the presence of SCs, spleen cells were stimulated with ConA for 48 h in the presence of SCs or MSCs. The intracellular levels of selected cytokines were determined by flow cytometry. As shown in Fig. 4, the percentage of CD4$^+$TNFα$^+$ (Fig. 4A) and CD4$^+$IL-2$^+$ (Fig. 4B) cells was decreased in a dose-dependent manner after cultivation with SCs and MSCs (Fig. 4B). This effect was more pronounced in the presence of SCs. A significant decrease in the proportion of CD4$^+$IL-17$^+$ cells was observed in spleen cells stimulated with ConA in the presence of MSCs; cultivation with SCs has no significant effect on the intracellular level of IL-17.

The Effect of SCs on Peritoneal Macrophages

To further investigate the potential of SCs to modulate the phenotype of immune cells, cells isolated from the peritoneal cavity of mice were cultivated in the presence or absence of SCs or MSCs for 48 h. Macrophages can change their phenotype between pro- (M1) and anti-inflammatory (M2) type according to the cytokine microenvironment [32]. As shown in Fig. 5, the percentage of F4/80$^+$cells positive for CD206, a marker of M2 macrophages, was significantly increased. In the presence of MSCs in the culture, no significant changes were detected.

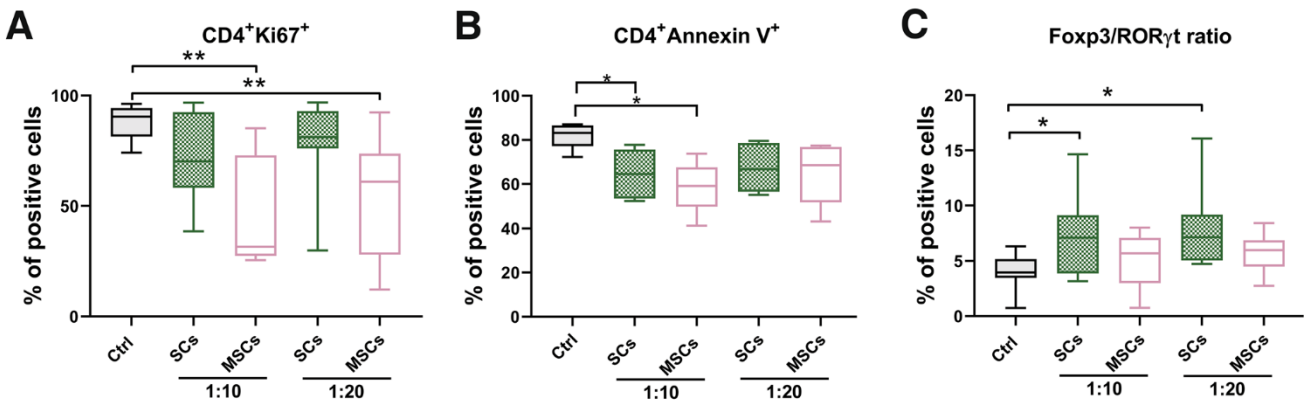


Fig. 3 The effect of SCs and MSCs on proliferation, apoptosis and Foxp3/RORγt ratio of CD4$^+$ T cells. Spleen cells were stimulated for 48 h (for apoptosis detection) or 72 h (for transcription factors and Ki67 determination) with ConA alone or with ConA in the presence of SCs or MSCs in various ratios. The proliferation of CD4$^+$ Tcells, as positivity of Ki67 **A** and the percentage of apoptotic CD4 + Tcells, as detected using Annexin V **B**, was determined by flow cytometry. Foxp3/RORγt ratio of CD4$^+$ T cells in co-cultures with MSCs or SCs was determined by flow cytometry **C**. Data are expressed as means ± SE from five independent experiments. Statistical significance between groups is marked with asterisks (*$p < 0.05$): Ctrl—control

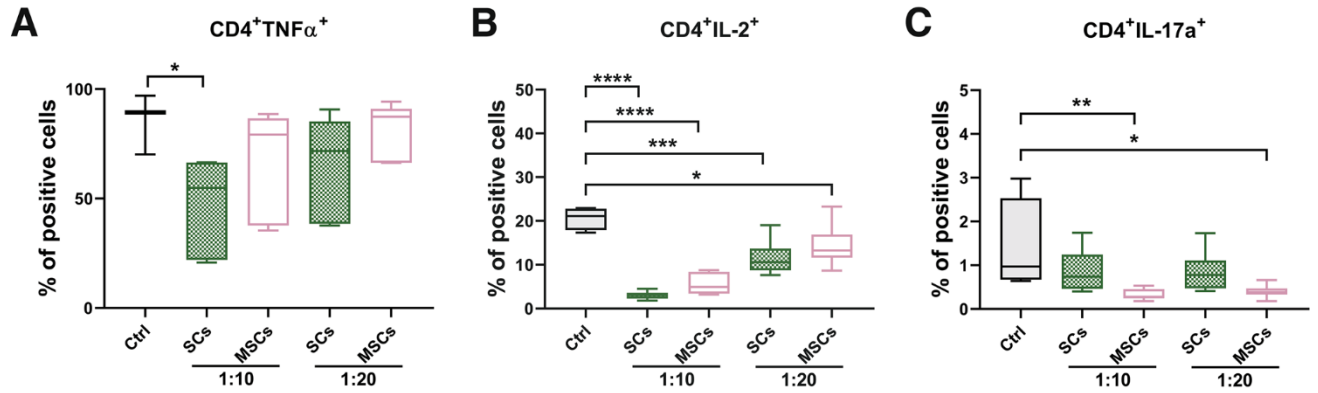


Fig. 4 The effect of SCs and MSCs on IL-2, TNFα and IL-17 producing $CD4^+$ T cells. The spleen cells were stimulated with ConA in the presence of SCs or MSCs for 48 h. The proportion of $CD4^+TNF\alpha^+$ **A**, $CD4^+IL\text{-}2^+$ **B** and $CD4^+IL\text{-}17^+$ **C** cells was determined by flow cytometry. Data are expressed as means ± SE from five independent experiments. Statistical significance between groups is marked with asterisks (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). Ctrl—control

The Ability of SCs to Transfer Mitochondria to Immune Cells

It is known that MSCs can transfer mitochondria to various types of cells and thus modulate their metabolism or phenotype [33, 34]. Therefore, we investigated the possibility of whether SCs are also able to transfer mitochondria to immune cells. Spleen cells were cultivated in the presence or absence of SCs or MSCs stained for mitochondria with MiTT. As shown in Figs. 6A and 6B, SCs possess a similar capacity to transfer mitochondria to immune cells as have MSCs, as determined by the percentage of $CD45^+MiTT^+$ cells in co-cultures of spleen cells with MSCs or SCs. Figure 6C shows fluorescence microscopy images of this experiment.

Discussion

MSCs are currently studied in many areas of regenerative medicine and developmental biology. It is increasingly apparent that the choice of the appropriate type of MSCs is crucial to the favourable outcome of therapy. MSCs isolated from various tissues have been used in cell-based therapies to promote the repair of testicular damage or treatment of male infertility. Although the results of these studies have been promising, further research in this area is needed [25, 35, 36]. In this regard, the use of cells possessing immunosuppressive properties, which occur naturally in affected testicular tissue, could be beneficial. SCs have been for a long time supposed to be nourishing cells providing support for germ cells, and creating a blood-testis barrier. Nowadays, this simple view has been expanded. SCs have been described to provide testicular immune privilege, regulate immune response and play an essential role in modulating the phenotype of immune cells, changing the environment within testes from tolerogenic into inflammatory in the presence of infection [37–39]. However, the immunoregulatory properties of SCs were tested mainly in co-transplantation studies [7, 17, 40]. A better definition and understanding of the stem capabilities of SCs will allow the extension of their therapeutic use for the regeneration of testicular tissues and the treatment of many other diseases.

Two previous studies suggested that SCs are kind of MSCs. However, these studies were based mainly on their

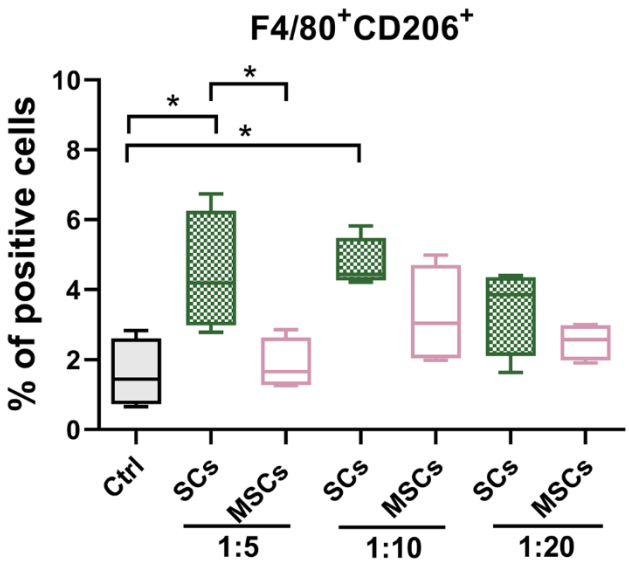


Fig. 5 The expression of CD206 marker on macrophages co-cultivated with SCs and MSCs**.** Peritoneal exudate cells were co-cultured with MSCs or SCs for 48 h, and the proportion of $CD206^+F4/80^+$ cells was determined by flow cytometry. The data are expressed as means ± SE from five independent experiments. Statistical significance between groups is marked with asterisks (*p < 0.05): Ctrl—control

morphological properties [8] and the ability to differentiate into key cell types of mesenchymal origin [10]. On the SC surface, we confirmed the expression of MSC- specific markers and the absence of hematopoietic markers as defined by Dominici et al. [30]. We further focused on the immunomodulatory abilities of SCs. MSCs induce arrest of the T cell cycle and thus suppress proliferation and apoptosis of these cells [41, 42], and promote polarisation into anti-inflammatory T cell populations [43, 44]. SCs demonstrated similar properties, although they differed in their expression. The ability of SCs to inhibit proliferation was less pronounced than that of MSC's; on the other hand, they increased the Treg /Th17 ratio even more than MSCs.

MSCs have been described to alter cytokine production by various immune cells populations [45, 46]. In this study, we have shown that SCs significantly suppressed the production of pro-inflammatory cytokine TNFα, and the down-regulation of IL-2 production by SCs was even more pronounced. On the other hand, the suppression of IL-17 was not significant in the case of SCs. According to our data, both cell types modulate cytokine production and suppress inflammation. However, MSCs seem to be more effective in suppressing Th17 response, while SCs suppress inflammation or activation of $CD4^+$ T cells by down-regulating the production of two key cytokines IL-2 and TNFα, respectively.

The effect of SCs on innate immune cells has been documented but never compared to MSCs before. The polarisation of macrophage, from pro-inflammatory M1 to wound-healing M2 population, depends greatly on the microenvironment and external stimuli [32]; macrophages could acquire alternative M2 phenotype also in the presence

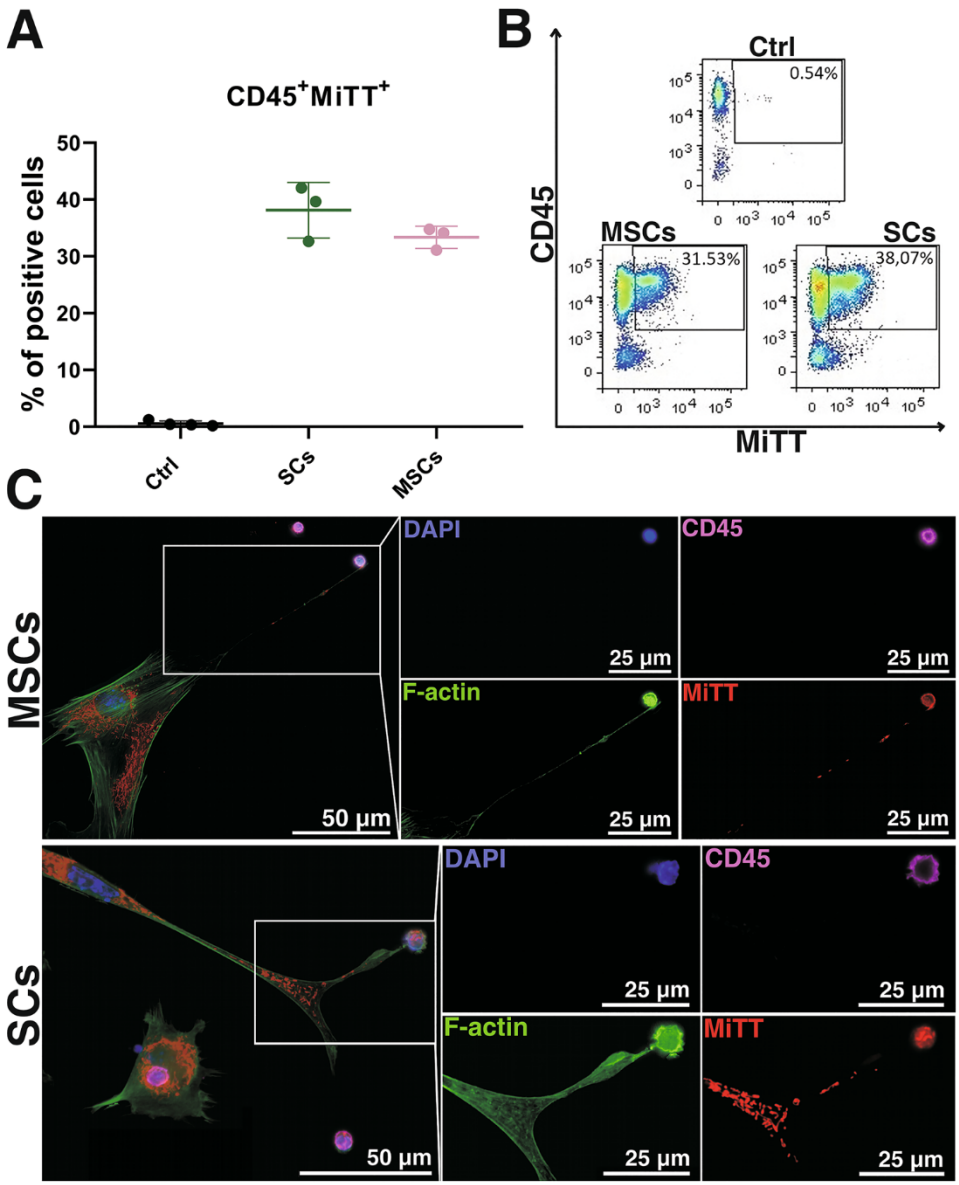


Fig. 6 The transfer of mitochondria from MSCs and SCs to $CD45^+$ immune cells. MSCs and SCs were stained by MiTT and cultivated with spleen cells for 3 h. The transfer of MiTT positive mitochondria originated from MSCs or SCs into $CD45^+$ cells was evaluated by flow cytometry **A**. One representative of the dot plot analysis from 3 independent experiments is presented **B**. The mitochondrial transfer was also documented by immunohistochemical detection. The insets show higher magnification of white squared regions in each channel (green -F-actin stained by phalloidin; red – MiTT, blue – nuclei; pink – mAb against CD45) **C**. Ctrl—control, MiTT—MitoTracker

of MSCs [28, 47, 48]. According to our data, SCs were more effective in the induction of this tolerogenic phenotype, as shown by up-regulation of CD206 molecule on their surface, which may subsequently extend their ability to regulate T cell immune responses. In the testes, the interplay between SCs and other cell populations, especially macrophages, is crucial for maintaining the optimal conditions for spermatogenesis and immune privilege function [7, 49]; this could be the reason why SCs showed better efficacy in inducing the M2 macrophage phenotype.

The direct link between a metabolic configuration of immune cells and their phenotype and function is well known and vastly studied [50]. The master regulators of the metabolic setup are mitochondria, and the transfer of this organelle between different cell types was reported as an inductor of a phenotype switch, polarisation or protection of recipient cells [51]. For example, the transfer of mitochondria from MSCs to T cells triggers Treg differentiation and repression of Th17 cells [20, 33]. MSCs modulate macrophage phagocytosis and activity also via mitochondrial transfer, both in vitro and in vivo [34, 52]. We showed here for the first time the ability of SCs to transfer mitochondria to other cell types. The percentage of $CD45^+$ immune cells, which acquired mitochondria from SCs and MSCs, was similar.

This study confirms the previously stated hypothesis about mesenchymal origin and stem cell-like properties of SCs. We have shown that SCs differentiate into key cell types of mesenchymal origin and express some MSCs-like markers. There is a growing body of evidence indicating the presence of a rare population of pluripotent stem cells, called very small embryonic stem cells (VSELs), in multiple adult tissues, including testis [26, 53]. They can differentiate into 3 germ layers and participate in testicular tissue regeneration and initiate testicular germ cell tumours [54, 55]. The microscopy and flow cytometry analysis revealed that 99 percent of cells used in our studies expressed SOX9, a marker of SCs (Supplementary Fig. S4). Although it has been reported that the pluripotent character of VSELs is manifested after 3- week culture by colony-forming [56], we did not observe colonies in either culture during differentiation. However, possible contamination of cell cultures isolated from testicular tissue by VSELS should be kept in mind.

Regarding their immunomodulatory properties, SCs are similar to MSCs, but differ in several aspects, which we assume can be attributed to their specific natural niche in the organism. SCs are in close contact with various cells in testes, provide support for germ cells, but also are responsible for a protective immune environment. In this respect, mitochondrial transfer could be one of the regulatory mechanisms. The obtained data support the possibility of applying SCs in various therapeutic approaches, especially testicular inflammation, orchitis or impaired spermatogenesis due to exposure to chemotherapy or radiotherapy. A better understanding of the effects of SCs and their capacity to down-regulate inflammation in vivo will be a crucial step for their implementation in cell-based therapy.

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Data Availability The authors confirm that all data and materials support the published claims and comply with field standards.

Declarations

Ethics Approval This study was carried out in strict accordance with the Act No. 246/1992 Coll., on the protection of animals against cruelty, the basic law related to animal protection governing the activities of all the state authorities of animal protection in the Czech Republic, such as the Ministry of Agriculture, including the Central Commission for Animal Welfare, and the veterinary administration authorities. The authorization to use experimental animals was issued to the Faculty of Science, Charles University, 37,428/2019-MZE-18134.

Conflict of Interest The authors declare that they have no conflict of interest.

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