Stem Cell Research & Therapy

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Therapeutic potential of Sertoli cells in vivo: alleviation of acute inflammation and improvement of sperm quality

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Abstract

Background Inflammation-induced testicular damage is a significant contributing factor to the increasing incidence of infertility. Traditional treatments during the inflammatory phase often fail to achieve the desired fertility outcomes, necessitating innovative interventions such as cell therapy.

Methods We explored the in vivo properties of intravenously administered Sertoli cells (SCs) in an acute lipopolysaccharide (LPS)-induced inflammatory mouse model. Infiltrating and resident myeloid cell phenotypes were assessed using flow cytometry. The impact of SC administration on testis morphology and germ cell quality was evaluated using computer-assisted sperm analysis (CASA) and immunohistochemistry.

Results SCs demonstrated a distinctive migration pattern, importantly they preferentially concentrated in the testes and liver. SC application significantly reduced neutrophil infiltration as well as preserved the resident macrophage subpopulations. SCs upregulated MerTK expression in both interstitial and peritubular macrophages. Applied SC treatment exhibited protective effects on sperm including their motility and kinematic parameters, and maintained the physiological testicular morphology.

Conclusion Our study provides compelling evidence of the therapeutic efficacy of SC transplantation in alleviating acute inflammation-induced testicular damage. These findings contribute to the expanding knowledge on the potential applications of cell-based therapies for addressing reproductive health challenges and offer a promising approach for targeted interventions in male infertility.

Keywords Sertoli cells, Inflammation, Macrophages, Testes, Sperm

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Introduction

Infertility and impaired fertility are significant health concerns, affecting approximately 15% of couples worldwide, with male factors contributing to half of the cases [1]. Approximately 15% of male infertility is linked to reproductive tract inflammation, characterized by leukocyte infiltration or systemic inflammatory conditions. Various infections, of the male genital tract can lead to the disruption of the blood-testis barrier (BTB), ultimately impairing sperm function [2]. In a study conducted by Gallegos-Avila et al., 78% of infertile men were tested positive for *Chlamydia trachomatis* or mycoplasmas [3]. *Chlamydia muridarum* infection in mice resulted in reduced sperm count, motility, and subsequently in infertility [4]. Furthermore, recent research by Bryan et al. (2020) has shown that this impact is passed on to offsprings [5]. In addition to a direct impact on germ cells, bacterial infections trigger the release of inflammatory mediators, including the production of reactive oxygen species (ROS) and pro-inflammatory cytokines by immune and somatic cells. This cascade of events not only compromises sperm viability but also attracts leukocytes, which phagocytose defective spermatozoa and disrupt the integrity of the BTB [6, 7]. Currently, patients suffering with orchitis are typically administered a standard antibiotic regimen and treatment for impaired reproduction is not specific, involving hormonal support, antioxidants to mitigate oxidative stress, and anti-inflammatory drugs [8]. Hence, it is imperative to devise advanced treatment approaches aimed at averting testicular damage and minimizing the loss of germ cells. Stem cells hold significant promise as a treatment option for various diseases. In particular, mesenchymal stem cells (MSCs) have attracted attention owing to their potent anti-inflammatory properties [9–12]. Within the seminiferous tubules, Sertoli cells (SCs) directly interact with developing germ cells. They establish the BTB, provide essential nutrients, and create a supportive environment for germ cell development [13]. Furthermore, SCs play a pivotal role in maintaining the immune-privileged status of the testicular environment, thereby contributing to its anti-inflammatory milieu [14, 15]. Apart from their function in the testes, SCs have exhibited remarkable immunoregulatory capabilities, positioning them as potential candidates for cell-based therapies in a spectrum of conditions, including diabetes, neurodegenerative diseases, and infertility as reviewed by Washburn et al. (2022) and Chiappalupi et al. (2024) [16, 17]. Notably, SCs transplantation successfully restored spermatogenesis in cases of busulfan-induced SC and germ cell deficiencies [18] or after irradiation [19]. However, the specific impact of SCs on bacterially induced testicular inflammation, particularly in relation to spermatogonia damage, remains unclear.

The exact mechanisms underlying the suppressive effects of SCs remain a subject of ongoing research. Recent studies have indicated that their capacity to modulate the immune system and suppress inflammation can vary based on the specific experimental models employed [14, 20]. SCs express various molecules that influence both innate and adaptive immune responses. TGF-β has been identified as a critical factor used by SCs in immune modulation. Its production leads to the induction of regulatory T cells (Tregs), either directly via the expression of indoleamine 2,3-dioxygenase (IDO), soluble JAGGED1, or indirectly via antigen presenting cells (APCs) [21, 22]. Extensive reviews are available for an in-depth exploration of the multifaceted nature of SC immunomodulation [16, 23].

Recently, in the article by Porubska et al. (2021), we compared the immunomodulatory capabilities of MSCs and SCs in vitro. Remarkably, our findings demonstrate that SCs share similarities with MSCs in this regard. Notably, SCs exhibit superior efficacy compared to MSCs in specific anti-inflammatory aspects. Specifically, we demonstrated an enhanced ability to induce the M2 macrophage phenotype, indicated by increased CD206 positivity. This distinctive proficiency may be attributed to the specialized niche of testicular environment that SCs occupy within an organism [24].

The present study sought to clarify the influence of SC application in a mouse model of LPS-induced testicular inflammation. The analysis focused on the inflammatory response, specifically examining key immune subpopulations implicated in this process. Notably, myeloid-derived immune cells, including macrophages, monocytes, and DCs, are the primary players in testicular immune surveillance [25]. Given that male pathologies compounded by infection often result in compromised sperm function and subsequent infertility, we assessed the sperm motility and overall fitness as well as testicular morphology. This evaluation will provide critical insights into the potential therapeutic effects of SCs in the alleviation of inflammation-induced associated reproductive dysfunction.

Materials and methods

Animals

Male BALB/c mice aged 8 to 12 weeks were used for the in vivo experiments. SCs were isolated from mice at 3 weeks of age. The animals were sourced from AnLab s.r.o. in Prague, Czech Republic. Ethical approval for this study was obtained from the Charles University Animal Ethics Committee (MSMT-25422/2022-4). All experimental protocols strictly adhered to established guidelines for the care and use of laboratory animals. Animals were kept under standard conditions of 22 ± 1 °C, 12-hour light/12-hour dark, and free access to water and food. The number of animals in a cage was determined based

on the area of the floor of each cage. The experimental animals were euthanized by cervical dislocation. Calypsol (containing ketamine – 100 mg/kg body weight) and Rometar (containing xylazine – 10 mg/kg body weight) were used for sedation. This study was reported in accordance with the ARRIVE 2.0 guidelines.

Isolation and preparation of Sertoli cells

SCs were isolated according to a previously described method [24]. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, PAA Laboratories, Pasching, Austria) supplemented with 10% FBS (Sigma-Aldrich Corporation, St. Louis, MO, USA), antibiotics (100 mg/mL streptomycin, 100 U/mL of penicillin) and 10 mM Hepes buffer, hereafter referred to as the complete medium. The cells were maintained as adherent monolayers, and passages 3 to 6 were used in the experimental procedures.

Experimental groups

The male mice (12w) were distributed randomly into three distinct groups as indicated in Fig. 1. Each group contained 3–4 animals per experiment. Following a seven-day acclimatization period, mice were randomly distributed into three categories, each corresponding to one of three experimental groups: control, LPS, and LPS + SCs. Each cage housed mice from all experimental groups. Upon administration of the injection, a number was assigned to each animal. The specific details regarding the experimental group and its respective number were only accessible to the researcher responsible for administering the injections. At the conclusion of the assessment period, each number was converted into the corresponding experimental group. This system ensured blinding and randomization during measurements. The first group consisted of naive animals assigned as the control group (Ctrl). They were subjected to intraperitoneal (i.p.) injections of phosphate buffer saline (PBS) followed by intravenous (i.v.) PBS injections the following day. The second group, denoted as the LPS group (LPS), received an i.p. injection of lipopolysaccharide (LPS, Sigma Aldrich, St. Louis, MO, USA) at a dosage of 3 mg/kg, to induce testicular inflammation [26]. After 24 h, the mice were i.v. injected with PBS. The third group, denoted as the SC group (LPS + SCs), received an i.p. injection of LPS and was the next day injected i.v. with SCs at a concentration of 1×10^6 in 100 µL of sterile PBS. After injections, mice were closely monitored, 48 h after the LPS injection, the mice were euthanized for further analysis.

Determination of SCs migration after LPS-induced inflammation

To study SC migration after induction of inflammation, male mice received LPS. After 24 h, SCs were labeled with the CellVue® Claret Far Red Fluorescent Cell Linker Mini Kit (CellVue, MINCLARET, Sigma-Aldrich) according to manufacturer´s instructions, and 1×10^6 cells/100 µl PBS was transplanted intravenously. Mice were euthanized 48 h after LPS injection, and various organs were harvested, and single cell suspensions were prepared. Cells were stained according to the standard flow cytometry protocol. Briefly, samples were incubated with antibodies (Supplementary Table S1) in 100 µL FACS solution for 30 min at 4 °C, followed by two washes with FACS solution. Dead cells were stained with propidium iodide (1:1000, PI, Exbio, Prague, Czech Republic). Analysis was performed on 3×10^5 live cells using

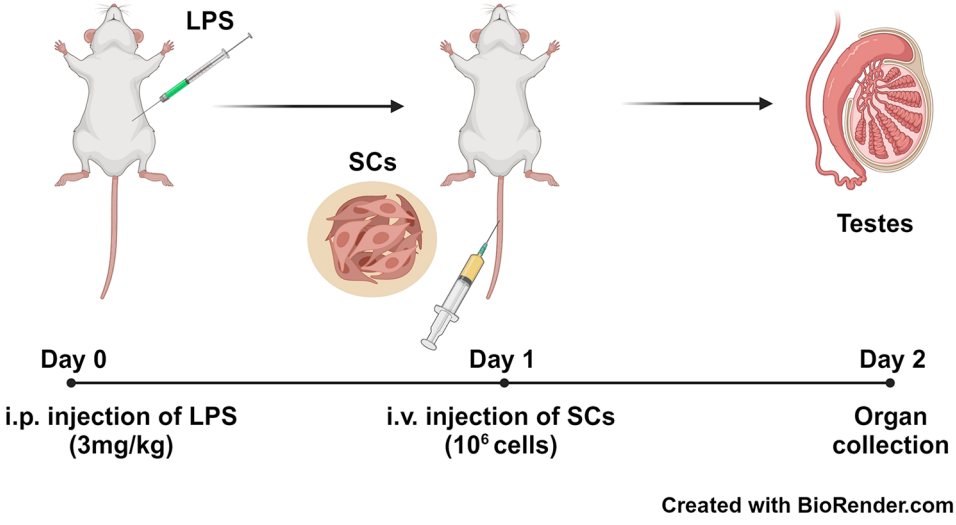

Fig. 1 Experimental design. LPS was injected intraperitoneally on day 0, followed by intravenous injection of SCs 24 h later. Mice were sacrificed 48 h after LPS injection and testes, epididymis or other organs were collected and processed

the FlowJo© software (FlowJo BD, Ashland, OR, USA). To assess the localization of SCs within the seminiferous tubules, the testes were cryosectioned, stained for nuclei, and mounted using Mowiol-DAPI (Sigma-Aldrich). Lysosomes were stained with LysoDye working solution (1 µmol/L, Dojindo Molecular Technologies, Kumamoto, Japan) in Hanks' HEPES buffer at 4 °C overnight. Alternatively, SCs were double stained with 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE, 5 µM, BioLegend, San Diego, CA, USA), and CellVue before application. The prepared samples were observed using a Leica Dmi8 fluorescence microscope (Leica Microsystems, Wetzlar, Germany). Data were analyzed using ImageJ software and images were deconvoluted with Huygens Professional version (23.10) using the "Standard" Deconvolution Express Profile with Acuity set to -80.0. (Scientific Volume Imaging, The Netherlands, http://svi.nl).

Detection of immune markers in testes by flow cytometry

A single-cell testicular suspension was prepared as follows. One testis per animal was cut multiple times with scissors and transferred into 500 µL of PBS. DNase I (0.05 mg/mL, Sigma-Aldrich) and Collagenase II (1 mg/mL, Sigma-Aldrich) were added to the samples, and the tissue was digested at 37 °C for 45 min. Subsequently, the suspension was filtered through a 40 µm filter, and transferred to 2 mL of FBS to stop enzyme activity. All subsequent steps were performed in Hank's balanced salt solution (HBSS, Sigma-Aldrich) supplemented with DNase I (0.02 mg/mL) and FBS (1%). Cell suspensions were incubated for 30 min on ice with conjugated monoclonal antibodies (see Table S1). Cells were washed twice, and 6×10^5 live cells were analyzed. Dead cells were stained with PI. Data acquisition was performed using BD Aria II (Becton Dickinson, BD, Franklin Lakes, NJ, USA) and analyzed using FlowJo© Software. A representative gating strategy is shown in Supplementary Fig. S1.

Cytokine detection within testicular tissue by ELISA

The testes were harvested to assess the effect of SCs on pro- and anti-inflammatory cytokines following LPS-induced inflammation. Cytokine detection was performed using ELISA for IL-1β (DY401, RD Systems, Minneapolis, MN, USA s), CCL1 (DY845, RD Systems), and IL-10 (DY417, RD Systems). Briefly, tissues were weighed, homogenized, subjected to three freeze-thaw cycles, and centrifuged. The supernatants were collected for subsequent cytokine evaluation according to the manufacturer's instructions.

Evaluation of sperm motility

Sperm motility was assessed using a computer-assisted sperm analysis (CASA) system. Initially, the cauda epididymis was collected, segmented into multiple pieces, and placed in 500 µL of M2 medium (M7167, Sigma-Aldrich) for a 10 min incubation at 37 °C with 5% CO_2. Subsequently, sperm were incubated for 60 min in 20 µL of M2 medium, covered with paraffin oil, at 37 °C to facilitate capacitation. Both capacitated and non-capacitated sperm samples were utilized for the assessment of sperm count and motility via the CASA system (ISAS v1, Proiser, Valencia, Spain). In detail, 2 µL of the sample was loaded onto a counting slide (Leja SC-20; 20 µm deep), and six fields were recorded for each sample. The fields were strictly located at the outer edges of the chamber to avoid the Segre–Silberberg effect. The following kinematic parameters were derived and analyzed by cluster analysis: curvilinear velocity (VCL, m/s), velocity of the average path (VAP, m/s), straight line velocity (VSL, m/s), straightness (STR, %), linearity (LIN, %), and amplitude of the lateral head displacement (ALH, m). The percentage of total motility (VAP > 10 m/s) and progressive motility (VAP > 10 m/s and STR > 45%) were evaluated based on the default settings of the CASA software. Sperm tracks were captured for each individual sperm with 50 frame rate per second.

Immunohistological analysis

Hematoxylin-eosin (H&E) and immunohistochemical staining were performed using established methods. Samples for H&E staining were prepared using the protocol described before with some modifications [27]. Briefly, testicular samples were fixed with Bouin's solution for 24 h at 4 °C, rinsed, dehydrated by increasing concentrations of ethanol and xylene and embedded into paraffin for microtome sectioning. Sections (10 µm) were prepared following the standard protocol [28]. H&E stained sections were employed to assess the morphology of seminiferous tubules, as described previously [29]. For immunohistochemical staining samples were prepared using established methods for cryosection preparation. Testicular tissue samples were embedded in Tissue-Tek® O.C.T. Compound and flash frozen on dry ice. Tissue sect. (8 µm) were prepared, and immunocytochemistry was performed as previously described [30]. In brief, the sections were fixed with 4% paraformaldehyde for 15 min, washed in PBS, permeabilized with 0.1% Triton X-100 in PBS for 15 min, blocked by 5% BSA in PBS containing 0.1% Triton X-100 for 60 min, and then incubated with primary antibody rabbit anti-DDX4 (1:500; Merck Life Science s.r.o.) at 4 °C overnight. After washing with PBS, the sections were incubated with secondary antibody (1:1000 Alexa FluorTM 594 goat anti-rabbit, Thermo Fisher Scientific) for 60 min and washed with PBS. The slides were then mounted with Mowiol-DAPI. Data were acquired using the Leica DMi8 wide-field fluorescence microscope. Data were analyzed using ImageJ

software and signal for DAPI was deconvoluted with Huygens Professional version (23.10) using the "Standard" Deconvolution Express Profile with Acuity set to: -80.0.

Statistical analysis

For significance evaluation and statistical calculations, the GraphPad Prism 8 program (GraphPad Software, San Diego, CA, USA) was used. All results are expressed as the mean ± standard deviation (SD). The statistical significance of the differences between the means of individual groups was calculated using student´s t-test in Fig. 2A and one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test in all other figures. *P-values* less than 0.05 were considered statistically significant.

Detailed processing of sperm motility data was done by *k*-mean cluster analysis in STATISTICA 10 (StatSoft, Czech Republic). The aim was to classify motile spermatozoa into distinct subpopulations by individual sperm kinematic parameters. These motile spermatozoa from both experimental groups and control were processed in one dataset. The euclidean distances algorithm was applied to variables such as STR, VAP, VCL, VSL, and ALH. The process involved 20 iterations to define three clusters or subpopulations of sperm. By considering the computed means of the selected variables, each individual spermatozoon was subsequently assigned to one of three specific subpopulations (clusters): slow locally motile, medium motile and fast progressive motile. Differences in sperm distribution in clusters were analyzed by two-way ANOVA.

Results

SCs selective migration to the testes and liver

Various tissues including spleen, lymph nodes, heart, lung, liver, and testis were collected to assess the systemic distribution of intravenously administered SCs. The migration of CellVue-stained SCs in LPS-challenged mice was determined by flow cytometry, with $CD45^{-}CellVue^{+}$ cells identified as live SCs within the tissues. A substantial proportion of these cells exhibited specific accumulation in the liver and, importantly, in the testes (Fig. 2A). Conversely, $CD45^{+}CellVue^{+}$ cells were detected in the lungs and liver of recipients (Fig. 2B), suggesting phagocytosis of SCs by immune cells in these organs. Importantly, microscopic analysis revealed that CellVue-positive cells were localized to the basement membrane of seminiferous tubules. In addition to macrophages, SCs in the testis have the ability to phagocytose cells [9]. To further investigate whether the signal observed in the microscopic analysis was specifically targeted to lysosomes, indicating engulfment of incoming SCs by present phagocytes, we stained lysosomes using the specific lysosome tracker Lyso Dye. While some CellVue signals are associated with lysosomes, there are also CellVue-positive cells that have a distinct localization (Fig. 2C). To confirm this finding, we double-stained SCs prior to application. Unlike CellVue, which stains lipidic regions in the membrane, CFSE is a pH sensitive, cell-permeable dye that emits green fluorescence. Therefore, engulfed cells are negative for CFSE. As shown in Fig. 2D, cells positive for both CellVue and CFSE were detected at the basement membrane of the seminiferous tubules.

SCs application alters the percentage of myeloid cells in testes

To investigate the effect of SCs transplantation on LPS-induced testicular inflammation, we used multicolor flow cytometry to assess relevant immune surface markers in the testis suspension 48 h after LPS injection. Intraperitoneal LPS injection did not alter the viability, or the relative abundance of $CD45^{+}$ immune cells in the testis at 24 h compared to control mice (Fig. 3A). However, LPS injection induced the infiltration of $Ly6G^{+}CD11b^{+}$ cells, and this infiltration was suppressed in LPS + SCs-treated mice (Fig. 3B), indicating attenuation of the acute inflammatory response.

While macrophages constitute the most abundant immune populations within the testis [31, 32], the percentage of $CD45^{+}CD11b^{+}F4/80^{+}$ cells remained unchanged between cohorts (Fig. 3C). Therefore, two most prominent macrophage populations, peritubular and interstitial macrophages, were investigated. These populations were distinguished based on the expression of MHCII and CD206 markers, with peritubular macrophages identified as $MHCII^{+}CD206^{-}$ and interstitial macrophages as $MHCII^{-}CD206^{+}$ [33]. As shown in representative density plots and quantified in graphs, significant changes in macrophage populations were detected in the LPS-treated animals. Both interstitial and peritubular macrophage populations decreased, while $MHCII^{+}CD206^{+}$ and $MHCII^{-}CD206^{-}$ cells increased. In contrast, when LPS challenge was followed by SCs treatment, subpopulations of interstitial and peritubular macrophages were preserved (Fig. 3D). A detailed analysis of the macrophage subpopulations revealed significant changes in the expression of MerTK, a molecule associated with the induction of immune tolerance in peripheral tissues [34, 35]. In interstitial macrophages, MerTK expression markedly decreased in animals treated with LPS, whereas the population of peritubular macrophages remained unchanged compared to the control group. Notably, in SC-treated animals, peritubular macrophages showed an increase in MerTK expression compared to both control and LPS-treated animals. In addition, the population of $MHCII^{-}CD206^{-}$ cells in LPS-treated animals showed a decrease in MerTK expression (Fig. 3E).

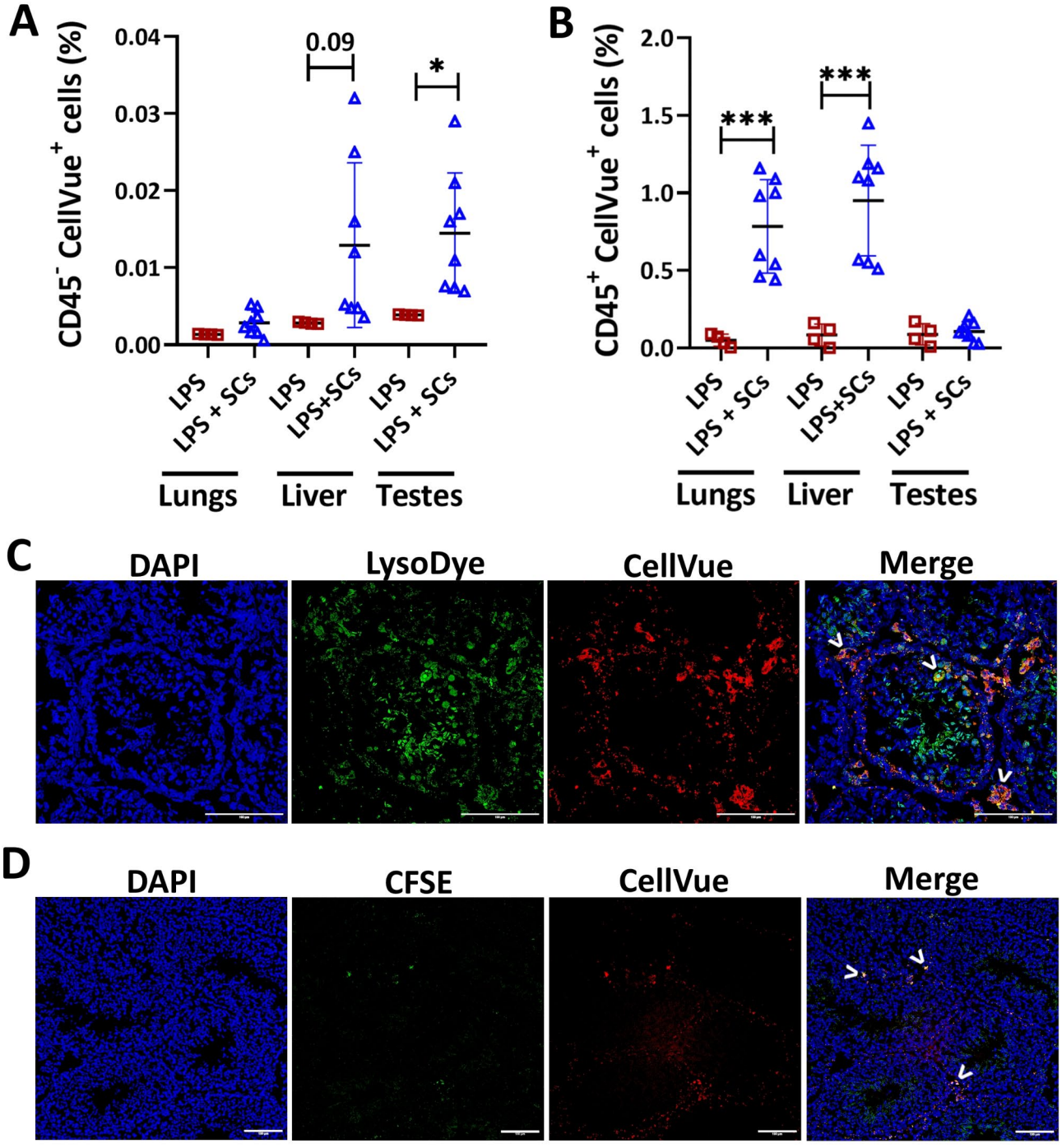

Fig. 2 Specific migration of SCs after intravenous application in LPS-treated recipients. **A** $CD45^-$ $CellVue^+$ SCs were found in liver and testis. **B** $CD45^+$ $CellVue^+$ cells considered as phagocytosed SCs in lung and liver. Data are presented as mean ± SD from 3 different experiments, $n = 8$. Statistical significance between groups is indicated by asterisks (*$P < 0.05$; ***$P < 0.001$), unpaired t test. LPS - lipopolysaccharide treated animals, LPS + SCs - animals treated with both LPS and SCs. **C** Representative microscopic image showing the localization of CellVue-positive cells in seminiferous tubules and degraded cells co-localized with lysosomes (white arrowheads show SCs). CellVue - red, Lyso Dye - green, DAPI - blue. **D** Representative microscopic image showing the location of CFSE, CellVue positive SCs and degraded SCs positive for CellVue (white arrowheads show SCs). CellVue - red, CFSE - green, DAPI - blue. Scale bar – 100 µm

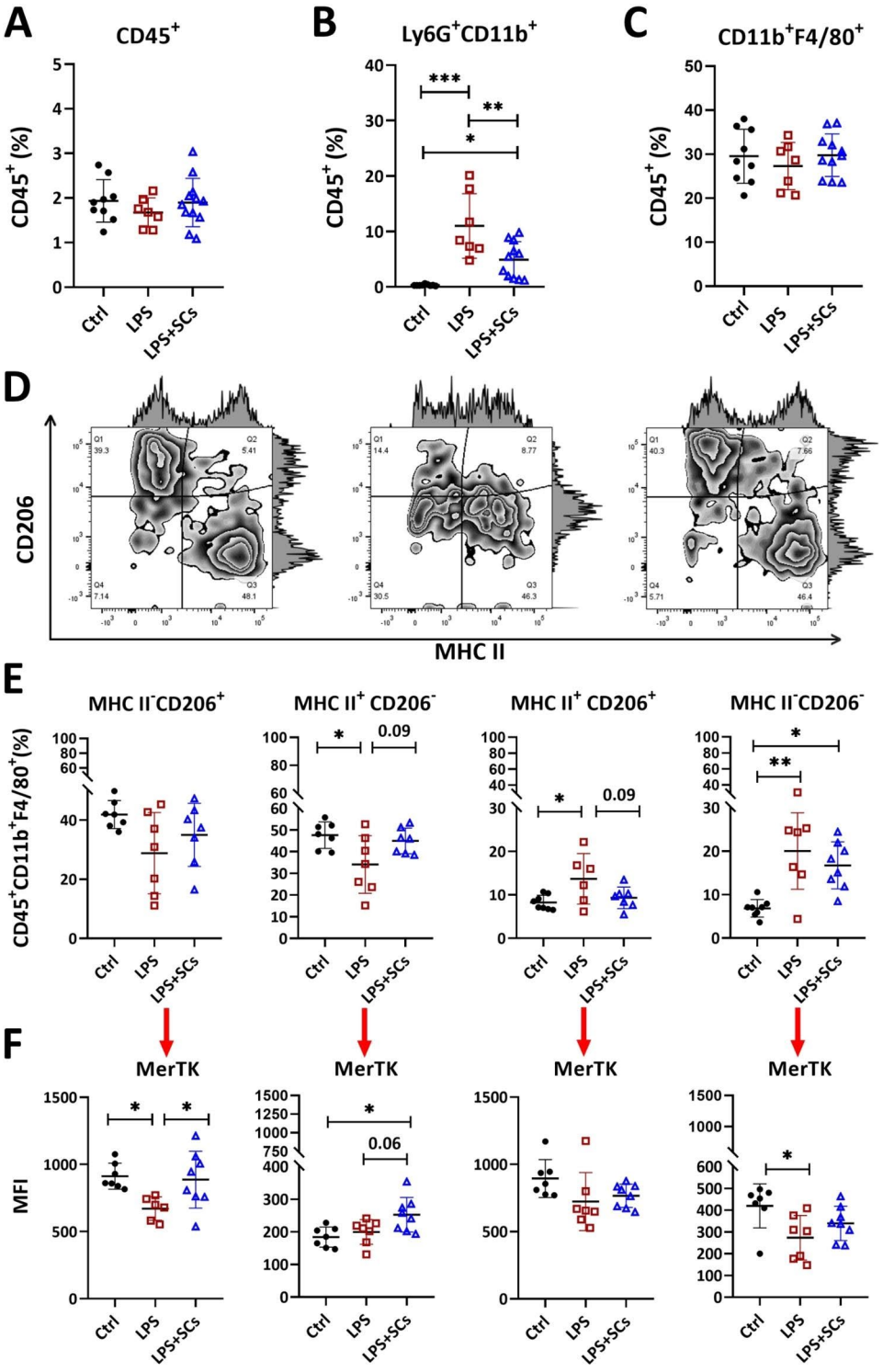

Fig. 3 Effect of SC administration on immune cell populations in the testes of LPS-treated recipients. The testicular cell suspension was analyzed by flow cytometry. **A** Percentage of $CD45^+$ cells in the testicular tissue of animals treated with LPS, LPS + SCs, or vehicle. **B** Percentage of $Ly6G^+CD11b^+$ cells from $CD45^+$ cells in the testes. **C** $CD11b^+F4/80^+$ cells from $CD45^+$ cells in the testes. **D** Representative density plots and **E** graphs showing macrophage subpopulations ($CD45^+CD11b^+F4/80^+$) distinguished by MHC II and CD206 expression. Data depict changes in distinct testicular macrophage populations in LPS-treated animals and LPS + SCs-treated animals. **F** MerTK expression on individual macrophage populations, macrophage populations and the respective MerTK expression are placed in the same column (see the red arrows). Data are presented as mean ± SD of three independent experiments ($n = 7–9$). Statistical significance between groups is indicated by asterisks (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$), one-way ANOVA followed by Tukey's multiple comparison test. Ctrl - control animals, LPS - lipopolysaccharide treated animals, LPS + SCs - animals treated with both LPS and SCs

SCs application alters testicular cytokine production

We further explored changes in the testicular cytokine milieu by analyzing testicular homogenates after the LPS application and subsequent SC treatment. Although testicular weight remained unchanged for 48 h post-inflammatory induction (see Supplementary Fig. S2), there were significant changes in production of specific cytokines. IL-1β levels increased after LPS application, and this trend persisted with SCs treatment. In contrast, SCs application effectively suppressed the LPS-induced production of CCL1, a cytokine released by activated M2 macrophages [36]. In addition, IL-10 production was significantly reduced after LPS injection, but this reduction was less pronounced in the SC-treated group (Fig. 4).

SCs application alleviate inflammation-related sperm motility impairment

Testicular inflammation has a detrimental effect on sperm fitness [37] particularly sperm motility. To assess the potential of SCs to ameliorate the effects of LPS-induced inflammation, we collected the cauda epididymis and analyzed sperm motility using CASA. The sperm count of LPS-treated animals was lower than that of the control or SC-treated animals (Fig. 5A). The number of immotile sperm increased after inflammation, but this effect was attenuated after SCs treatment. While sperm motility before capacitation exhibited similar levels between groups, following capacitation, a decreased motility of sperm isolated from animals treated with LPS alone was revealed. In contrast, sperm from the SC-treated animals displayed motility comparable to that isolated from the control animals, indicating the beneficial effect of SCs treatment on fertility during acute inflammation (Fig. 5B-D). Results of more in-depth analysis of sperm motility by clustering the motile sperm to sub-populations and taking to account the heterogeneity of the sperm sample, revealed no significant differences ($P > 0.05$) between control and experimental groups. However, in the LPS experimental group after the capacitation, there was increase in the slow locally motile cluster and decrease in fast progressive motile cluster. Importantly, in the group treated with SCs, the percentage of sperm in fast progressive motile cluster increased to a similar level as in the control group (Fig. 5E).

SCs application preserve testicular morphology

To evaluate changes in testicular architecture, testes were excised from untreated and treated groups and testicular sections were stained with H&E. Microscopic analysis revealed disruption of testicular morphology, expansion of interstitial regions between seminiferous tubules, and a slight reduction of elongated spermatids in the LPS-treated group. Notably, the detrimental effects observed in the LPS-treated group were alleviated after SCs application, as indicated by improved testicular morphology and number of elongated spermatids comparable to the control (Fig. 6). Microscopic images also showed a weaker expression of DDX4 in the LPS-treated group (Fig. 7). DDX4 expression is localized to germ cells. The increased signal of DDX4 staining in the SC-treated group further supports the positive impact of SCs application on germ cell quality and testicular morphology (Fig. 7).

Discussion

Infections play a significant role in the rising prevalence of infertility. Presently, the treatment administered during the inflammatory phase frequently falls short in accomplishing the intended goal of restoring fertility. Biological interventions, especially cell therapy, are emerging as promising strategies for various pathologies. Although MSCs are widely used, their widespread application in

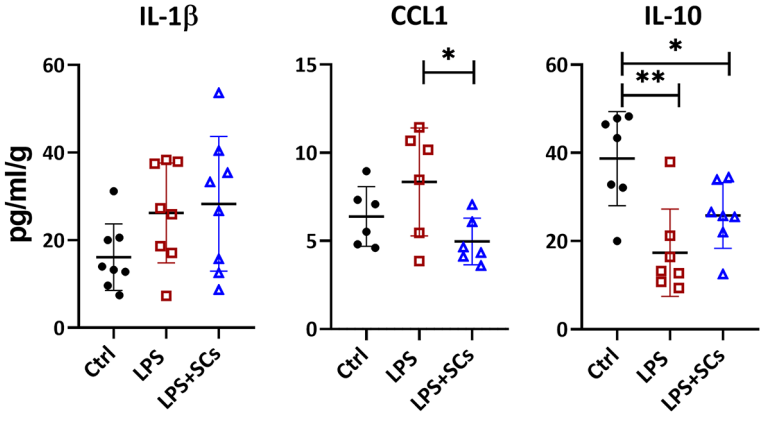

Fig. 4 Effect of SC administration on cytokine production in testicular tissue homogenate from LPS-treated recipients. Testicular homogenates were prepared, and various cytokines, including IL-1β, CCL1 and IL-10 were detected by ELISA. Data are presented as means ± SD from three independent experiments (n =6-8). Statistical significance between groups is marked with asterisks (*P < 0,05, **P < 0,01), one-way ANOVA followed by Tukey's multiple comparison test. Ctrl - control animals, LPS - lipopolysaccharide-treated animals, LPS + SCs - animals treated with both LPS and SCs

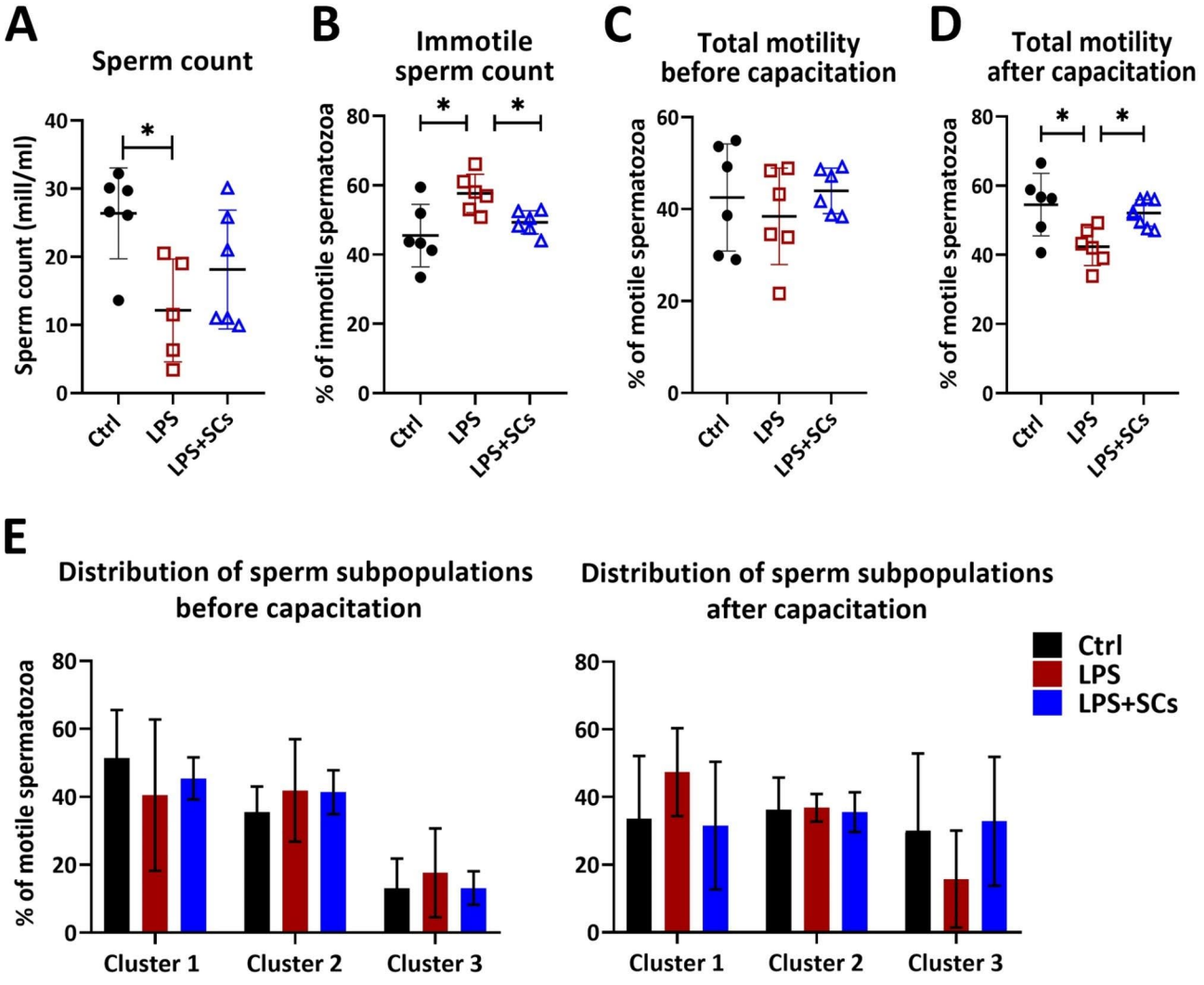

Fig. 5 Effect of SC administration on sperm quality in LPS-treated recipients. **A** Sperm count, **B** number of immotile sperm, and total motility **C** before, and **D** after capacitation was determined using a computer-assisted sperm analysis (CASA) system. **E** Sperm percentage in individual clusters (1 – slow locally motile, 2 – medium motile, 3 – fast progressive motile) before and after capacitation. Data are presented as mean ± SD of three independent experiments ($n = 5$-7). Statistical significance between groups is denoted with asterisks (*$P < 0.05$), one-way ANOVA followed by Tukey's multiple comparison test. Ctrl - control animals, LPS - lipopolysaccharide treated animals, LPS + SCs -animals treated with both LPS and SCs

clinical settings is hampered by issues such as internal heterogeneity and low engraftment rate [38, 39]. It has been recently postulated that SCs possess immunomodulatory properties similar to MSCs [24, 40]. In order to acquire a more comprehensive understanding of the in vivo characteristics of SCs, we introduced fluorescently labeled SCs intravenously to mice 24 h following inflammation induced by LPS. Our initial focus was to determine a distribution of SCs within the body. Fluorescence-positive cells, expressing CD45, indicating potentially phagocytized SCs, were detected in the lungs and liver. This distribution differed slightly from the typical pattern observed with MSCs after intravenous injection, as these SC-associated cells were absent in all lymphoid tissues [41]. Noteworthy, we observed CD45-negative SCs specifically present in the testicular tissue, suggesting a distinct migration pattern to the tissue of origin. In addition, SCs were detected at the basement membrane of the seminiferous tubules, demonstrating the potential for SCs transplantation into SC-deficient/damaged testes via the intravenous route, avoiding a potential risk of compartment syndrome from local injection [42, 43]. A significant proportion of the signal from transplanted SCs co-localized with the lysosome signal, suggesting that these SCs were engulfed. However, some cells exhibited a distinct localization pattern, which was confirmed by double staining with CSFSE and CellVue dyes, indicating the presence of live cells, a finding consistent with the flow cytometry data. With a relevance to this research, previous findings indicate that the phagocytosis of MSCs

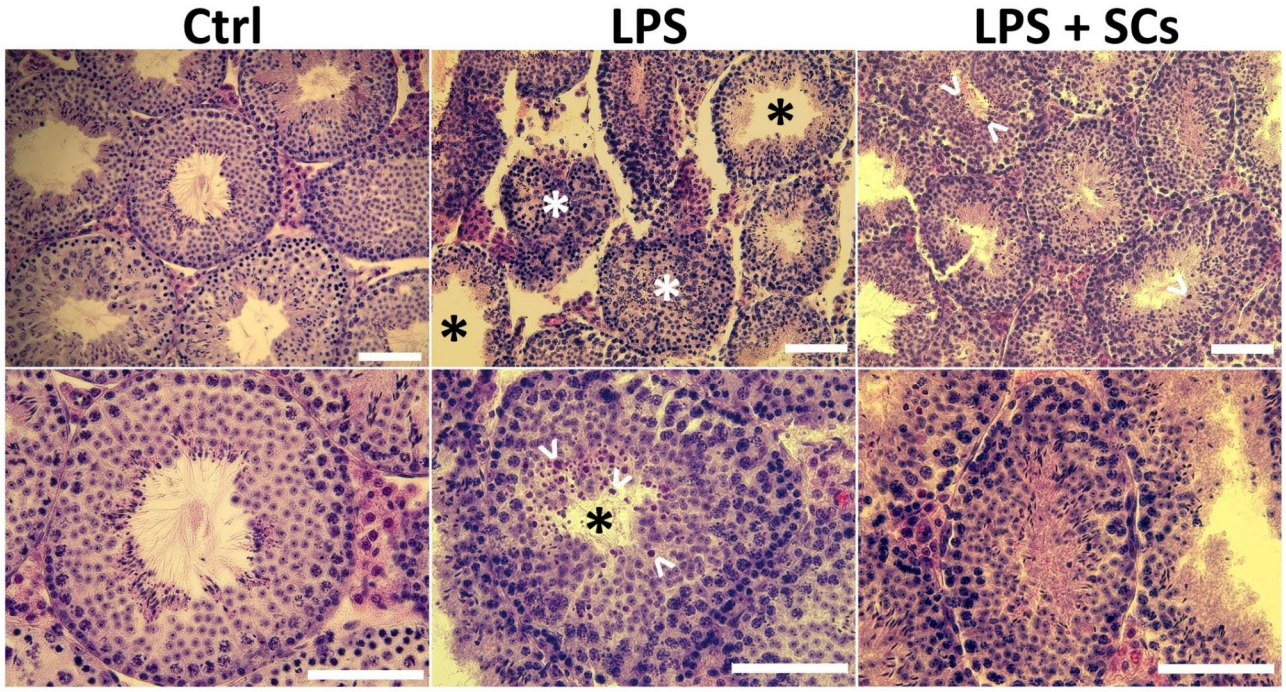

Fig. 6 Effect of SC administration on testicular morphology in LPS-treated recipients. Representative histological image of testicular tissue samples from the three groups studied, stained with hematoxylin-eosin. Image shows changes in the morphology of seminiferous tubules and interstitial regions in LPS-treated animals and LPS + SCs-treated animals. Black asterisks mark seminiferous tubules with slight reduction of elongated spermatids; white asterisks mark disorganized seminiferous tubules; white arrowheads show atypical residual bodies. Ctrl - control animals, LPS - lipopolysaccharide treated animals, LPS + SCs - animals treated with both LPS and SCs. Scale bar – 100 µm

can prompt a transition to the M2 phenotype in macrophages [37]. Consequently, it is reasonable to speculate that a comparable phenomenon might occur with SCs. The testicular microenvironment contains a number of immunomodulatory molecules, including testosterone, corticosterone, prostaglandins and activin, which have the potential to regulate the phenotype and function of immune cells in the testis [25]. Testicular macrophages maintain a predominantly M2 phenotype, even when activated by LPS [44]. Neutrophils, as early responders to inflammation, are recruited to the inflamed testis, and contribute to pathogen clearance [45]. In a study by Zhong et al. (2020), pretreatment with human umbilical cord-derived MSCs demonstrated a reduction in neutrophil infiltration during testicular ischemia/reperfusion injury [42]. Similarly, in our model, neutrophil infiltration increased after LPS injection, but this response was alleviated in animals treated with SCs.

Early resolution of inflammation, including the efficient clearance of tissue-damaging neutrophils, is essential for the proper repair of damaged tissue. Macrophages play a pivotal role in both the resolution of inflammation and tissue repair and remodeling. This is particularly significant in the testes, where macrophages constitute the most abundant immune cell population [31]. During infection-induced injury, macrophages rapidly engulf dying neutrophils through efferocytosis, a process critical for limiting inflammation. MerTK, a receptor tyrosine kinase, plays a central role in efferocytosis, particularly in the recognition and engulfment of apoptotic cells by phagocytes [46]. This molecule plays a key role in testes as the absence of MerTK disrupts spermatogenesis [35].

In our model, the control group exhibited distinct populations of interstitial and peritubular macrophages, characterized by differential expression of CD206 and MHCII. However, following LPS injection, there was a significant increase in the percentage of $CD11b^{+}F4/80^{+}$ $MHCII^{-}CD206^{-}$ macrophages. Given that classical blood monocytes typically exhibit low MHC II expression and do not express CD206 [47–50], it is plausible to speculate that this population reflects inflammation-induced monocyte infiltration. Remarkably, the $MHCII^{-}CD206^{-}$ population was reduced after the application of SCs. The intensity of MerTK fluorescence varied between groups. Notably, in both interstitial and peritubular macrophages, SCs application significantly increased the expression of MerTK, a receptor associated with the clearance of apoptotic cells, resolution of inflammation, and prevention of chronic inflammation [35]. This improvement may be related to the beneficial effects of SCs in modulating the testicular microenvironment.

CCL1 is a chemokine produced by activated M2 macrophages and plays a role in the recruitment of monocytes, natural killer (NK) cells, and DCs [51]. The suppressed

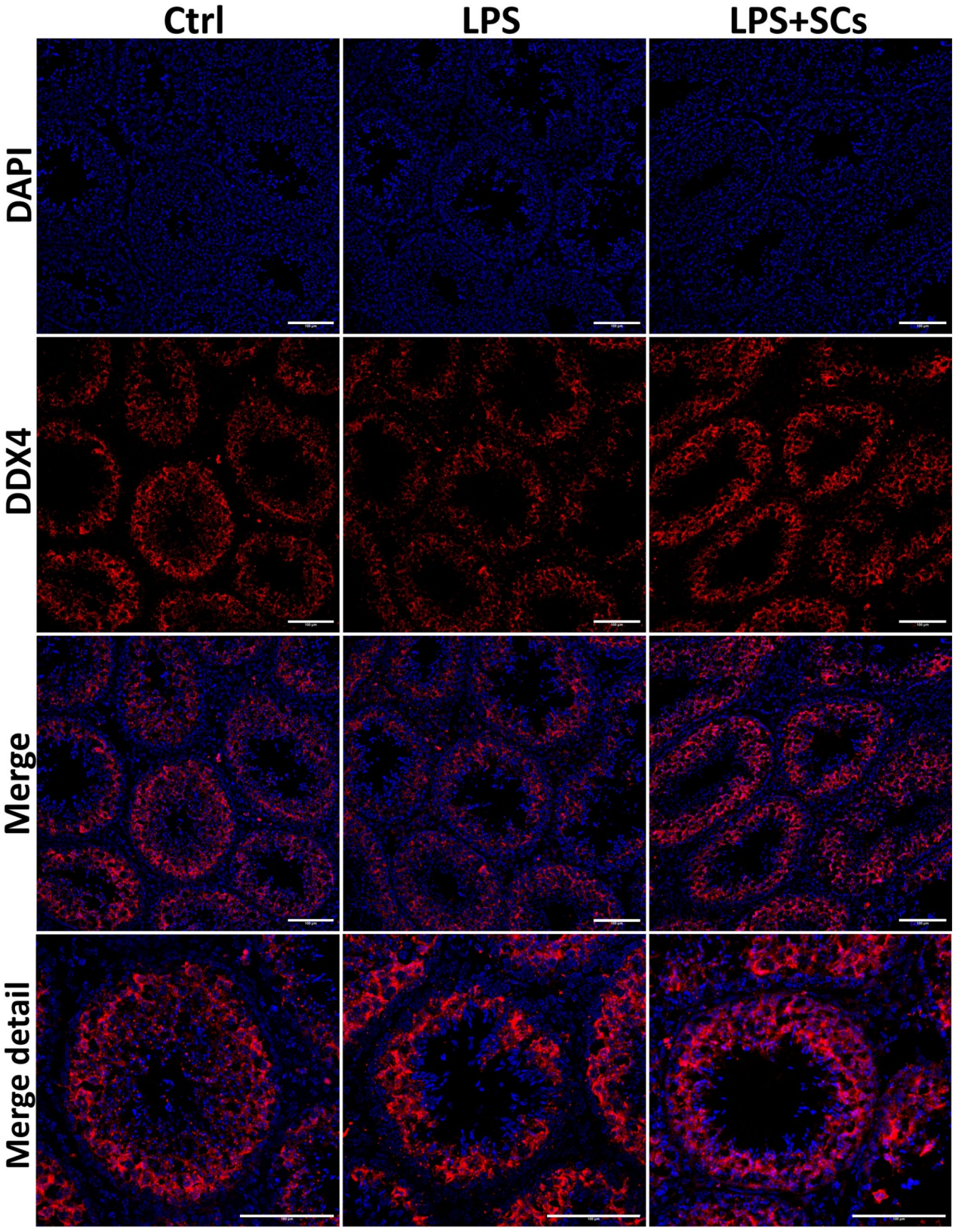

Fig. 7 Effect of SC administration on germ cell quality in LPS-treated recipients. Representative images of testicular sections stained for DDX4-positive cells. DDX4 - red, DAPI - blue. Ctrl - control animals, LPS - lipopolysaccharide treated animals, LPS + SCs - animals treated with both LPS and SCs. Scale bar – 100 µm

production of CCL1 after SC application, compared to LPS treatment alone, may reflect differences in the macrophage subpopulations present in testes between these two groups. As M2 macrophages are a significant source of IL-10 in the testis [52], it is important that production was elevated after the SCs application, IL-10, partially counteracting its suppression during LPS-induced inflammation. This reinforces the data obtained by flow cytometry, that SCs contribute to restoring the balance of M2 macrophage-associated cytokines. On the other hand, aside from macrophages, other immune cells in the testis, including SCs, were described to produce IL-1β in response to inflammatory stimuli [53]. This is consistent with the observation that IL-1β production did not differ between the LPS-only and LPS-and-SC-treated groups.

In correlation with knowledge, that inflammation has a potential to adversely affect sperm production [37], LPS-induced inflammation resulted in a substantial decrease in sperm count and motility in our model. However, it is noteworthy that SCs application had a beneficial effect, effectively restoring motility. In our study we used more in-depth statistical approach by cluster analysis which better reflects sperm heterogeneity [54]. After capacitation, an increase of fast progressive motile sperm, which reflect potentially the highest fertilizing ability [55], showed positive effect of the treatment by SCs. Further, the morphology and functionality of the seminiferous tubules are critical determinants of future sperm fitness and perturbations in this microenvironment, whether due to inflammation, or other factors, can have profound effects on the quality and health of spermatozoa [29, 56]. The inflammation induced by LPS had a negative impact on the morphology of the seminiferous tubules. Additionally, the expression of DDX4, a germ cell marker, was lower than that of the control group. This suggests that testicular inflammation has a harmful effect on germ cells, which in turn affects the quality of sperm. Our findings also suggest that the observed sperm damage was not limited to epididymis but was associated with testicular inflammation. SC application was associated with preservation of testicular morphology and expression of DDX4. These findings highlight the positive impact of SCs in attenuating the detrimental effects of inflammation on sperm parameters.

Limitations and future directions

Inflammation can cause significant damage in many aspects of human life. Therefore, new approaches, including cell therapies [57] or drugs of natural origin [58–60] need to be further investigated. Although this study elucidated the therapeutic effects of SCs in testicular inflammation, the potential limitation of this study is its conduction on a mouse model. Further studies need to be carried out using Sertoli cell transplantation in order to confidently assess its positive outcome in humans. Nevertheless, multiple pre-clinical studies have outlined the benefits of Sertoli cell transplantation in multiple animal models rendering positive outcomes regarding multiple diseases [61]. Furthermore, the xenotransplantation of Sertoli cells was proven to effectively modulate the immune system of the host [62–64] showcasing promising results for future human transplantations. Future research should therefore focus on the identification of suitable sources of SCs for transplantation. These may include biopsies from men undergoing TESE surgery [65], cadaveric testes [66], or xenogeneic Sertoli cells [63, 67].

Conclusions

In conclusion, our study highlights the promising role of SC transplantation in alleviating inflammation-induced testicular damage, subsequently supporting fertility in men suffering from various infections. SCs exhibited unique tissue homing capabilities, specifically accumulating in the testicular tissue. Transplanted SCs showed immunomodulatory effects, reducing neutrophil infiltration, and increasing MerTK expression in macrophages. Notably, SC application exerted protective effects on sperm parameters, preserving sperm count, and motility and kinematics. In addition, SCs preserved seminiferous tubule morphology. This study provides valuable insights into the therapeutic potential of SC transplantation for the treatment of inflammation-induced testicular damage, offering a promising approach for targeted interventions in male infertility. A number of pre-clinical studies have demonstrated the potential benefits of SC transplantation in a range of animal models, with positive outcomes observed in the treatment of a number of diseases. Nevertheless, further studies are necessary to assess the efficacy of SC transplantation in humans with greater confidence.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13287-024-03897-9.

Supplementary Material 1

Acknowledgements

We are grateful to Dr. Jana Petrusova (Institute of Molecular Genetics of the Czech Academy of Sciences, Prague, Czech Republic) for her advice on testicular sections.

Author contributions

BP, MP and MK designed the study; BP, MP, NF, DV, VS, and OS performed experiments; OSi performed cluster analysis of motility data and interpreted the results; BP, MP and MK analyzed data; MK, BP, MP and TT prepared the manuscript, OSi, VK and KK critically revised the article; NF, MK, and KK provided funding acquisition. All authors read and approved the final manuscript.

Funding
This study was supported by grant No. 176524 from the Grant Agency of Charles University, grant No. NU21-08-00488 from the Ministry of Health of the Czech Republic, Institutional support of the Institute of Biotechnology, Czech Academy of Sciences (RVO 86652036), and BIOCEV (CZ.1.05/1.1.00/02.0109) from ERDF.

Data availability
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declaration

Ethics approval and consent to participate
The experimental procedures involving live animals were performed 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 and they also adhered to ARRIVE guidelines 2.0. This study protocol was approved by the Ethical Committee of Faculty of Science, Charles University and the Ministry of Education, Youth and Sports. Title of the approved project: Therapeutic potential of Sertoli cells and mesenchymal stem cells in mouse model of infertility treatment. Approval number MSMT-25422/2022-4. Date of approval: February 2, 2023.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Received: 29 January 2024 / Accepted: 26 August 2024
Published online: 04 September 2024

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