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RESEARCH ARTICLE

Granulocyte-macrophage colony-stimulating factor (GM-CSF)-induced maturation of spermatogonial cells from prepubertal mice *in vitro* is enhanced by testosterone

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ABSTRACT. Spermatogenesis is the complicated process of sperm generation. During this process, spermatogonial cells proliferate and differentiate via meiotic and post-meiotic stages to produce mature sperm. This process is under the regulation of testicular autocrine/paracrine factors. In addition, endocrine factors are crucial to complete spermatogenesis. We aimed to localize granulocyte-macrophage colony-stimulating factor (GM-CSF) and its receptor (GM-CSFR) in testicular cells and further evaluate its involvement in the development of spermatogenesis *in vitro*. We isolated cells from seminiferous tubule cells of seven-day-old mice and cultured them *in vitro* using a methylcellulose culture system (MCS), in the presence of GM-CSF and/or testosterone for four weeks. The cells were then examined for markers of different stages of spermatogenesis by immunofluorescence staining and/or qPCR analyses. Our results revealed the presence of GM-CSF and GM-CSFR in testicular cells (premeiotic and meiotic cells as well as somatic cells; Leydig and Sertoli cells). We further demonstrated the development of colonies/spheroids in the MCS which contained pre-meiotic, meiotic, and post-meiotic cells. The addition of GM-CSF to the MCS significantly increased the percentage of pre-meiotic and meiotic cells compared to control. Furthermore, the addition of GM-CSF and testosterone together significantly increased the percentage of cells in the post-meiotic stage compared to the addition of each separately. In conclusion, our results indicate that testicular cells express GM-CSF/GM-CSFR, and that GM-CSF is involved in the development of different stages of spermatogenesis *in vitro*. Furthermore, testosterone enhances the development of spermatogenic cells and potentiates the effect of GMCSF on the development of post-meiotic cells. These findings provide evidence that GM-CSF and testosterone are involved in the development of spermatogenesis *in vitro* and *in vivo*.

In brief: Testicular somatic and germ cells express GM-CSF and GM-CSFR. Our study suggests that testicular GM-CSF is involved in the development of spermatogenesis, which is potentiated by testosterone.

Key words: GM-CSF, testosterone, spermatogenesis, spermatogonial stem cells, *in vitro* differentiation of spermatogonial cells

Spermatogenesis is the process of the development of testicular germ cells (spermatogonia) into mature sperm cells. Spermatogonial cells divide and differentiate to produce primary spermatocytes, secondary spermatocytes, spermatids, and eventually spermatozoa. This process takes place in the seminiferous tubules that together with the interstitial tissue constitute the testicles. Each stage of cell development exhibits specific markers, such as: vasa, CDH-1, PLZF, and others in pre-meiotic cells; boule, CREM-1 and others in meiotic cells; and acrosin, protamine, and others in post-meiotic cells [1-11]. The function of the testicle is under endocrine regulation, and is affected by the hypothalamus and pituitary gland. The endocrine effects on the testicle are mediated and regulated at the level of the testicle by local control mechanisms - paracrine and autocrine factors [12].

LH acts primarily on testicular Leydig cells and binds to its specific receptor on these cells to increase the conversion of cholesterol to testosterone [12-15].

Testosterone affects the functionality of Sertoli and peritubular cells through the androgen receptor (AR). Testosterone diffuses through the plasma membrane and interacts with AR. AR then binds to androgen response elements (AREs) in gene promoter regions and

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recruits coregulator proteins that regulate gene transcription in those cells [16-18].

Testosterone plays a key role in the meiotic process during spermatogenesis in rodents. It is involved in oxidative metabolism, DNA repair, and RNA processing [16]. It affects the survival and maintenance of spermatogonial cells as well as their differentiation to meiotic stages [19, 20].

FSH affects different functions in Sertoli cells by increasing cAMP which subsequently increases the production of androgen binding protein (ABP), inhibin, and many other proteins. In addition, it plays an essential role in DNA synthesis in both the mitotic and meiotic stages of spermatid cells in rodents [12, 13].

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a glycoprotein that is typically known as a stimulator of maturation of hematopoietic cells from stem cells of the bone marrow, and has a broad role in modulating the innate immune response. It is secreted by macrophages, T cells, endothelial cells, and fibroblasts [21-23]. GM-CSF has been shown to be expressed in testicular macrophages [24] as well as testicular germ cells in humans [25, 26].

The GM-CSF receptor (GM-CSFR) is expressed in hematopoietic cells including progenitor and immune cells, as well as non-hematopoietic cells [25]. In addition, studies have shown that GM-CSFR is expressed in male germ cells, from spermatogonia to spermatids [25, 26]. Moreover, GM-CSF was also shown to affect the viability of porcine spermatogonia type (A) cells *in vitro* [27, 28].

One of the approaches to preserve male fertility is the establishment of *in vivo* and *in vitro* systems for the generation of fertile sperm [29-34]. Recently, our group established a novel 3-dimensional (3D) *in vitro* methylcellulose culture system (MCS) using methylcellulose as a scaffold, and demonstrated the induction of spermatogonial cells *in vitro* (from mice, monkeys, and humans) at different stages of spermatogenesis [29, 35-39].

In the present study, we examined the cellular localization of GM-CSF and its receptor in mouse testicular cells and evaluated the effect of GM-CSF on the development of spermatogenesis *in vitro* using the MCS. In addition, we investigated the role of testosterone in spermatogenesis *in vitro* in the presence of GM-CSF.

Our results may deepen our understanding of the role of the testicular GM-CSF system in *in vivo* and *in vitro* spermatogenesis.

MATERIALS AND METHODS

Animals

This study was performed in accordance with the Guiding Principles for the Care and Use of Research Animals Promulgated by the Society for the Study of Reproduction, and approved by the Ben-Gurion University Ethics Committee for Animal Use in Research (No. IL-16-04-2018). ICR mice at different ages (1-8 weeks old) were used (Harlan Laboratories, Jerusalem, Israel).

Immunofluorescence staining of mouse testicular tissue

Immunofluorescence staining of 4-μm thick sections from formalin-fixed, paraffin-embedded testicular tissue blocks was performed, as described previously [40]. Polyclonal rabbit anti-mouse GM-CSF antibody (17762-1-AP, Proteintech Europe United Kingdom; concentration: 700 μg/mL; final dilution: 1:500) or polyclonal rabbit anti-mouse GM-CSFR antibody (ab202851 Abcam; final dilution: 1:200) were used as primary antibodies. A fluorescent antibody (Cy-3, Jackson Immuno Research; donkey anti-rabbit antibody; final dilution: 1:1000) was used as a secondary antibody and the staining was performed as described previously [41].

Double staining was performed for GM-CSF and GM-CSFR in Sertoli cells (using vimentin as a specific marker- a goat polyclonal antibody: sc-7557, Santa-Cruz; concentration: 100 μg/mL; final dilution: 1:50), Leydig cells (using 3β-hydroxysteroid dehydrogenase [3βHSD] as a specific marker- a goat polyclonal antibody: Sc-30820, Santa-Cruz; concentration: 100 μg/mL; final dilution: 1:100), pre-meiotic cells (using CDH-1 as specific marker- a goat polyclonal antibody: AF748, R&D System; concentration: 100 μg/mL; final dilution: 1:1001) and meiotic cells (using boule as a specific marker- a goat polyclonal antibody: LS-B7334; LifeSpan BioSciences; final dilution: 1:133). A fluorescent antibody (Alexa Fluor 488; donkey anti-goat antibody; Jackson Immuno Research; final dilution: 1:500) was used as secondary antibody for immunofluorescence staining to determine the cellular origin of GM-CSF/GM-CSFR, as described previously [39].

Immunofluorescence staining of cell culture

Immunofluorescence staining was performed on cells isolated from culture after four weeks and fixed with methanol. Polyclonal rabbit anti-mouse vasa antibody (NBP224558, Novus Biologicals, Centennial, CO, USA; final dilution: 1:200), polyclonal rabbit anti-mouse PLZF antibody (SC-22839, Santa-Cruz; final dilution: 1:100), polyclonal goat anti-mouse boule antibody, (LS-B7334; LifeSpan BioSciences; final dilution: 1:133), and anti-acrosin antibody (NBP2-14260, Novus Biologicals; final dilution: 1:2000) were used as primary antibodies. The slides were incubated overnight at 4°C and then washed with PBS. The relevant fluorescent secondary antibodies were then added. Fluorescent antibodies, Alexa Fluor 488 (donkey anti-goat antibody; final dilution: 1:500; Jackson Immuno Research) and Cy-3 (donkey anti-rabbit antibody; final dilution: 1:1000; Jackson Immuno Research), were used as secondary antibodies and the staining was performed as described previously [42].

Extraction of total RNA for real-time PCR analysis

Extraction of RNA was performed using the Dynabeads RNA direct kit (Dynal Biotech, Oslo, Norway), as described previously [40]. Real-time quantitative PCR amplification of total cDNA (500 ng/sample) was performed using the following specific primers:

GAPDH:
Fw-5'ACCACAGTCCATGCCATCAC
Rw-5'CACCACCCTGTTGCTGTAGCC

Vasa:
Fw-5'AGTATTCATGGTGATCGGGAGCAG,
Rw-5'GCAACAAGAACTGGGCACTTTCCA

PLZF:
Fw-5'AGCTTGAAATACGTGGCCAGA
Rw-5'TGAGCAGTTCACACTTCATCCC

Boule:
Fw-5'AACCCAACAAGTGGCCCAAGATAC
Rw5'CTTTGGACACTCCAGCTCTGTCA

Acrosin:
Fw-5'TGTCCGTGGTTGCCAAGGATAACA
Rw-5' AATCCGGGTACCTGCTTGTGAGTT

GM-CSF:
Fw-5'ACCACCTATGCGGATTTCAT
Rw-5'TCATTACGCAGGCACAAAAG

GM-CSFR:
Fw-5'GTGTCAVGVTGGATGTGTCAAT
Rw-5'CAATGCTGTGTGTGATGACG

GDNF:
Fw-5'GCCCCTGCTTTCTATCTGCT
Rw-5'AGCCTTCTGAATGCGTGGTT

ABP:
Fw-5'-GCAGCATGAGGATTGCACTA
Rw-5'CATGAGGCTGGGGAATGTCT

AR:
Fw-5'TTGGGTGTGGAAGCATTGGA
Rw-5'TGGCGTAACCTCCCTTGAAA

Reactions were performed using Absolute qPCR SYBR Green mix (ABgene House, Blenheim Road, Epsom, UK), as described previously [40]. The results were expressed as fold increase relative to GAPDH for each sample.

Isolation of seminiferous tubular cells

Testicles from 6-7-day-old mice were surgically removed. The detailed protocol has previously been described [35]. In brief, tubules were enzymatically dissociated. The enzymatic digestion solution consisted of 1 mg/mL collagenase and 1 mg/mL DNAse. Both collagenase and DNAse were dissolved in PBS. The isolated cells were filtered through a sterile cell strainer (70 μM; BD Biosciences) in order to isolate the cells from the remaining debris. PBS was added and the suspension was centrifuged for 10 minutes at 1500 RPM. After centrifugation, we added 1 mL of medium (StemPro with 10% Knock Out serum (KSR). The total number of viable cells was counted under a light microscope (approximately 1×10^6/cells from each mouse; in each experiment, 10 mice were used).

Culture of isolated seminiferous tubular cells in vitro *using the methylcellulose culture system (MCS)*

Isolated cells were cultured in 24-well plates. Each well contained 200,000 cells in a total volume of 500 μL. For each well, the culture included 42% methylcellulose (R&D systems, Minneapolis, MN, USA) in medium consisting of 33% StemPro-34 medium, 10% KSR (knock-out serum replacement; Gibco, USA), and different growth factors, such as human rEGF (recombinant epidermal growth factor; Biolegend, San Diego, CA, USA) (20 ng/mL), human rGDNF (glial cell line derived nerve growth factor; Biolegend) (10 ng/mL), human rLIF (leukaemia inhibitory factor; Biolegend) (10 ng/mL), and human r-bFGF (basic fibroblast growth factor; Biolegend) (10 ng/mL).
In addition, GM-CSF (0.1-1 ng/mL) (Biolegend, San Diego, CA, USA) or testosterone (10^{-7} M) (Bayer, Hod Hasharon, Israel), alone or in combination, were added to the cultures. The cells were incubated for four weeks in a CO_2 incubator at 37 C.
At the end of the incubation, cells were collected, as described previously [43], and fixed for immunostaining to evaluate the stages of spermatogenesis, or kept at −70 C in a lysis solution to be used for RNA extraction, to examine the expression levels of markers of the stages of spermatogenesis.

Data handling and statistical evaluation

Each experiment was repeated at least three times. In *figures 1, 3 and 4*, the data is expressed as mean ± SEM (standard error of the mean). Analysis to determine statistical significance was performed using the unpaired Student's *t*-test and *p* value below 0.05 was considered significant.

RESULTS

GM-CSF and GM-CSFR are present in testicular cells and their expression increases with age

The cellular localization of GM-CSF was examined in testicular tissue of adult mice by double immunofluorescence staining using specific antibodies to GM-CSF and each cell type. Our results show that GM-CSF is localized to Sertoli cells, Leydig cells, spermatogonial cells (CDH1-positive cells), and meiotic cells (boule-positive cells) (*figure 1A*). The same positive staining was also observed for GM-CSFR in the same cell types (*figure 1B*).
Our results also show that the expression level of GM-CSF in testicular homogenates of mice of different ages (1, 2, 4, and 8 weeks) significantly increased with age, and was significantly higher in testicular homogenates of four-week-old mice compared to all other ages, based on qPCR analysis (*figure 1C*). The expression level of GM-CSFR also significantly increased with age in mice testicular homogenates of different ages (1, 2, 4, and 8 weeks), as examined by qPCR analysis, (*figure 1D*).

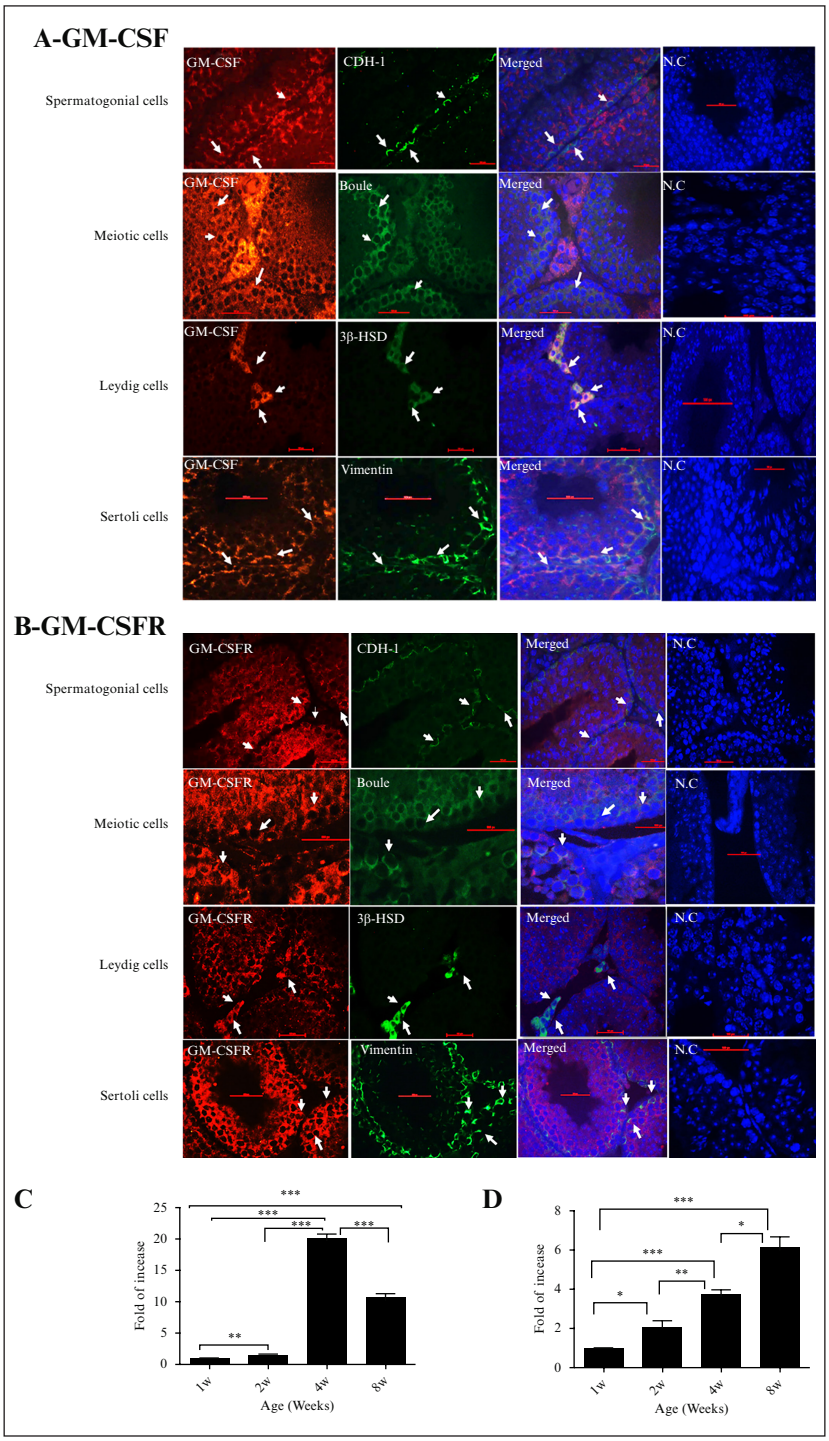

Figure 1.
GM-CSF and GM-CSFR are present in testicular cells, and their expression increases with age. **A**, **B**) Testicular tissues from mature mice (five-week-old) were analysed by immunofluorescence for GM-CSF and GM-CSFR as well as CDH-1, boule, 3β-HSD and vimentin. GM-CSF (**A**) and GM-CSFR (**B**) were localized to Sertoli cells, Leydig cells, spermatogonial (CDH1-positive) cells, and meiotic (boule-positive) cells (arrows). **C**, **D**) qPCR analysis of GM-CSF (**C**) and GM-CSFR (**D**) in testicular homogenates of mice at different ages (1, 2, 4, and 8 weeks). Testes from 10 mice were used for each age examined. *p<0.05, **p<0.01, ***p<0.001, based on comparison between different ages.

Development of clusters and cells of different stages during spermatogenesis in vitro

Based on our *in vitro* system using isolated cells from seminiferous tubules cultured in the MCS , we observed the development of clusters in cultures grown in the presence and absence of GM-CSF which did not appear to significantly differ in size or number (*figure 2A*). Moreover, in cultures grown in the absence or presence of GM-CSF, we observed the presence of cells of the pre-meiotic, meiotic, and post-meiotic stages, compared to cells before culture that stained only for the pre-meiotic stage (*figure 2B*).

Testosterone potentiates GM-CSF-induced spermatogenesis in vitro

The effect of testosterone and GM-CSF on spermatogenesis *in vitro* was investigated using cultured isolated seminiferous tubule cells in the MCS. Culture for four

weeks showed a significant increase in the percentage of pre-meiotic (vasa-positive) cells compared to before culture (*figure 3A1*). Furthermore, the addition of GM-CSF (0.1 ng/mL and 1 ng/mL) for four weeks significantly increased the mRNA level of pre-meiotic cell markers (vasa and PLZF), in a dose-dependent manner, compared to control (*figure 3A2, B*). Addition of testosterone during the latter two weeks did not significantly affect the percentage of vasa-stained cells compared to control, which was also unaffected in the presence of additional concentrations of GM-CSF (0.1 ng/mL and 1 ng/mL) (*figure 3A1*). However, the addition of testosterone significantly increased the mRNA level of vasa and PLZF cell markers, compared to control (*figure 3A2, B*, respectively). In the presence of 0.1 ng/mL GM-CSF, testosterone did not affect the mRNA level of vasa, however, in the presence of 1 ng/mL GM-CSF, testosterone significantly decreased the mRNA level of vasa and PLZF (*figure 3A2, B*, respectively).

Culture for four weeks also showed a significant increase in the percentage of meiotic/post-meiotic (boule/acrosin-positive) cells, compared to before culture (*figure 3C1 and D1*, respectively). Addition of GM-CSF (0.1 ng/mL and 1 ng/mL) for four weeks significantly increased the percentage of meiotic (boule-positive) cells (figure C1, D1) as well as the mRNA level of the meiotic (boule) cell marker in a dose-dependent manner (*figure 3C*), but not the percentage of post-meiotic cells or the post-meiotic marker (acrosin), compared to control (*figure 3D*). The addition of testosterone in the latter two weeks significantly increased the percentage of boule-stained cells and the mRNA level of boule compared to control (*figure 3C*), respectively). However, in the presence of different concentrations of GM-CSF (0.1 ng/mL and 1 ng/mL), the addition of testosterone (after two weeks of culture) did not affect the percentage of boule-stained cells or the mRNA level of boule, compared to GM-CSF alone (*figure 3C*). On the other hand, addition of testosterone after two weeks of culture significantly increased the percentage of acrosin-stained cells but not the mRNA level of acrosin compared to control (*figure 3D*). However, in the presence of different concentrations of GM-CSF (0.1 ng/mL and 1 ng/mL), addition of testosterone after two weeks of culture significantly increased the percentage of acrosin-stained

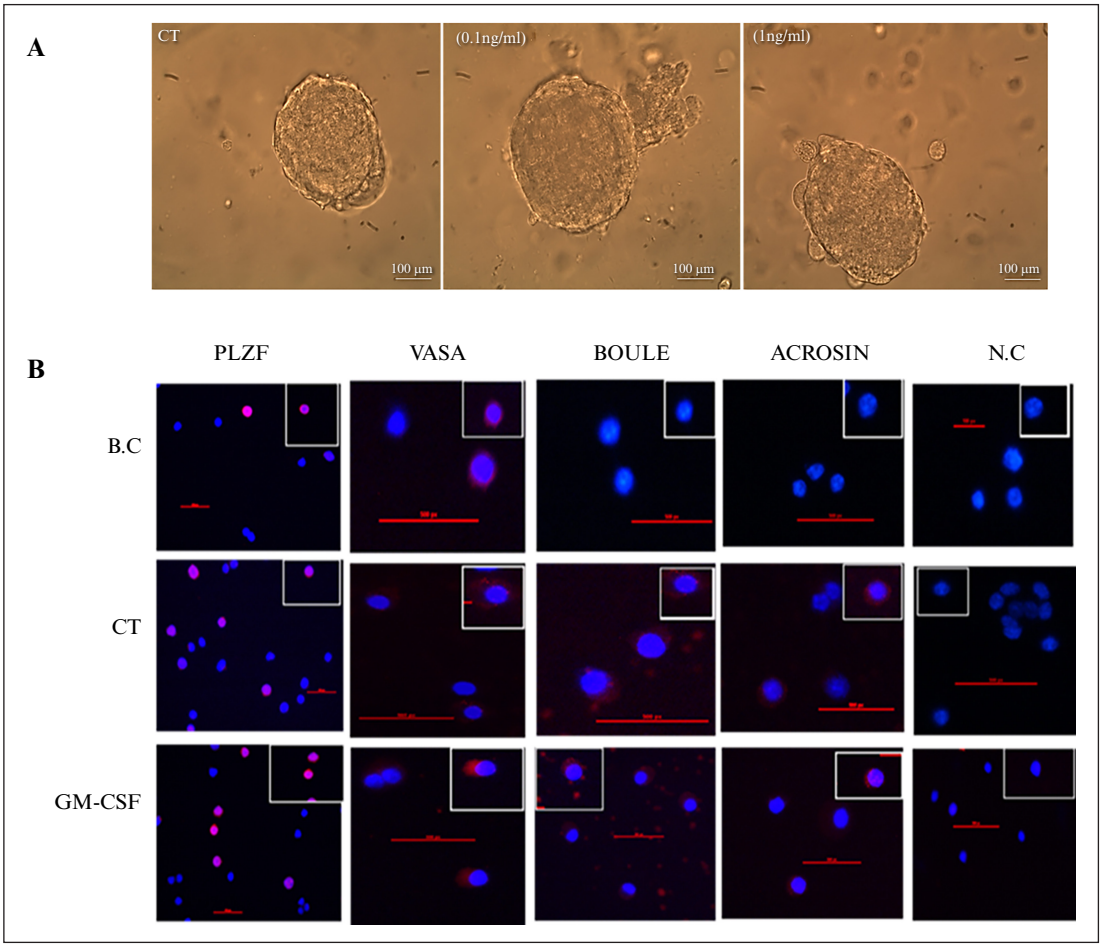

Figure 2.
Development of clusters and cells at different stages of spermatogenesis *in vitro*. **A)** Clusters from isolated cells of seminiferous tubules cultured in the MCS. Cells were isolated from seminiferous tubules of mouse testes (5-7 days) and cultured for four weeks in methylcellulose (as a 3D culture), containing StemPro Medium and growth factors (as described in the materials and methods section), in the absence or presence of different concentrations of GM-CSF (0.1-1 ng/mL). **B)** Cells and clusters/colonies were collected after four weeks of culture and fixed and stained with antibodies specific to cells of different stages of spermatogenesis using pre-meiotic markers (vasa, PLZF), a meiotic marker (boule) and a post-meiotic marker (acrosin). DAPI (blue) was used for nuclear staining. NC: negative control (without the first antibody); BC: before culture; CT: control (without GM-CSF). The experiment was repeated four times, each with 6-8 wells per treatment.

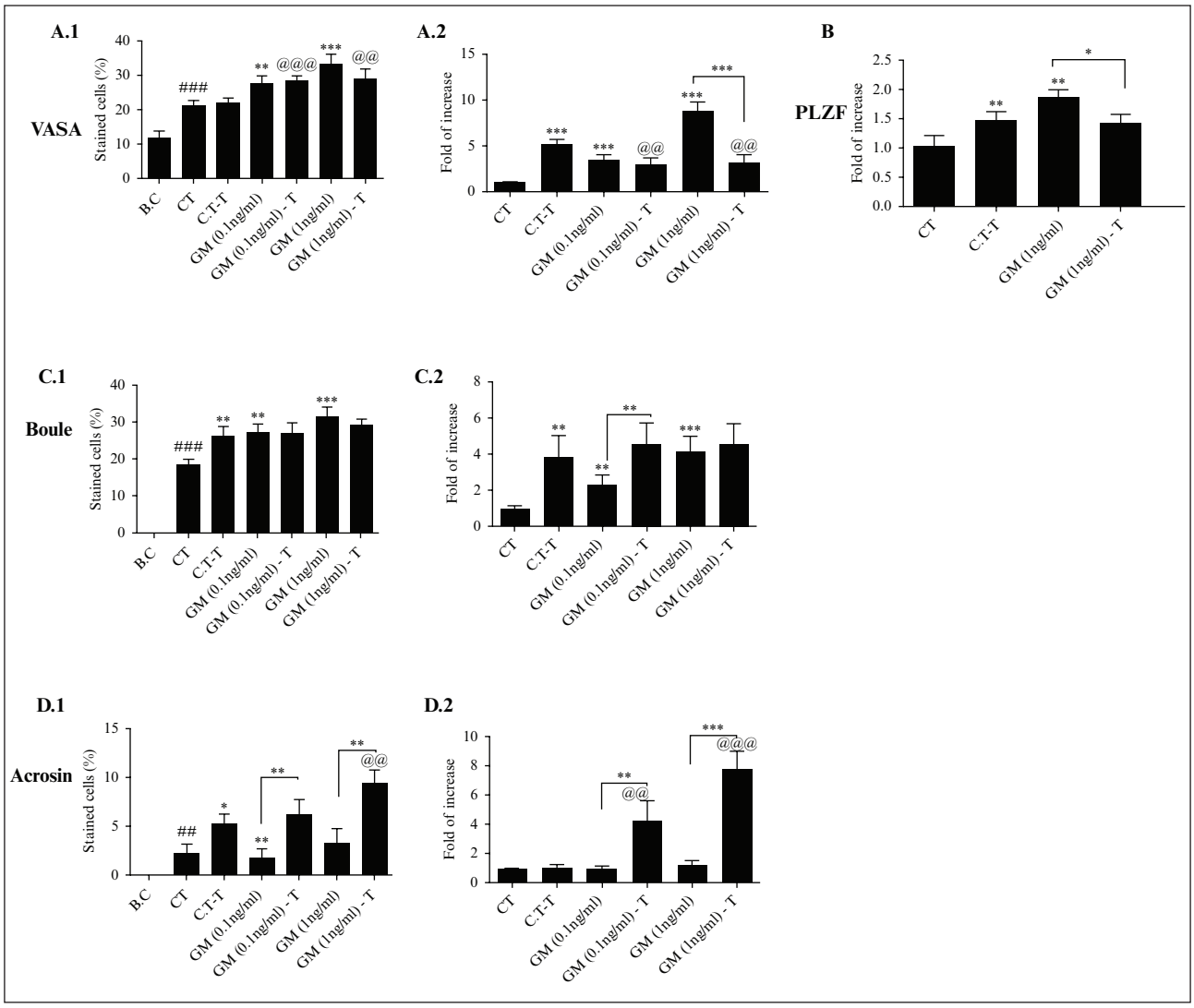

Figure 3.
GM-CSF-induced spermatogenesis is enhanced by testosterone *in vitro*. Isolated cells from the seminiferous tubules of immature mice were cultured in the MCS, containing StemPro Medium and growth factors, in the absence (CT) or presence (GM) of GM-CSF (0.1-1 ng/mL). After two weeks of culture, fresh media (CT or GM-CSF) was added, an in some wells, testosterone (10^{-7} M) was added to the CT group (CT-T) or GM-CSF group (GM-T) for a further two weeks of culture. Cells and colonies were collected after four weeks of culture. Cells were either stained with anti-vasa (**A1**), anti-boule (**C1**), or anti-acrosin (**D1**) antibodies to determine the percentage of cells at the different stages of spermatogenesis, or prepared for RNA extraction in order to evaluate the mRNA level of vasa (**A2**), PLZF (**B**), boule (**C2**) and acrosin (**D2**) by qPCR analysis. The experiment was repeated four times, each with 6-8 wells per treatment. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$; $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$, $^{@}p<0.05$, $^{@@}p<0.01$, $^{@@@}p<0.001$; based on comparison before culture (#), with CT (*) or CT-T (@).

cells as well as the mRNA level of acrosin, compared to GM-CSF alone (*figure 3D*).

Differential effect of GM-CSF and testosterone on Sertoli cell markers in vitro

The addition of testosterone to isolated cells from seminiferous tubules of seven-day-old mice, after two weeks of culture in the MCS, significantly increased the mRNA level of ABP and GDNF, but not AR, compared to control (*figure 4*). However, the addition of GM-CSF (1 ng/mL) after two weeks of culture did not significantly affect the expression level of ABP, AR and GDNF, compared to control (*figure 4*). On the other hand, addition of testosterone and GM-CSF (together) after two weeks of culture significantly increased the expression level of AR, but not ABP or GDNF, compared to addition of testosterone alone over two weeks (*figure 4*).

DISCUSSION

Our results demonstrate the expression of GM-CSF and its receptor (GM-CSR) in testicular mice cells. This includes both somatic cells (Sertoli, Leydig cells) and germ cells at different stages of their development, *i.e.* the pre-meiotic and the meiotic stages. Previous studies have also shown that GM-CSF is expressed in testicular macrophages and spermatozoa [44]. Our results may suggest that GM-CSF serves as a testicular paracrine/autocrine factor that is involved in the regulation and development of testicular functions, such as spermatogenesis.

In addition, we have shown that the expression level of GM-CSF and GM-CSFR increased significantly with

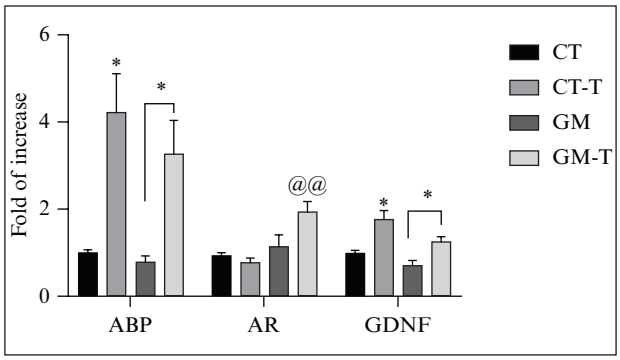

Figure 4.
The effect of GM-CSF and testosterone on Sertoli cell markers *in vitro*. Isolated cells from the seminiferous tubules of immature mice were cultured using the MCS, in the absence (CT) or presence of GM-CSF (GM) (1 ng/mL) with or without testosterone (T) (10^{-7} M), as described in *figure 3*. Cells/colonies were then collected after four weeks of culture, and RNA was extracted to determine the level of AR, ABP, and GDNF mRNA by qPCR. The experiment was repeated three times, each with 6-8 wells per treatment.*$p<0.05$; $^{@@}p<0.01$; based on comparison with CT (*) or CT-T (@). AR: androgen receptor; ABP: androgen binding protein; GDNF: glial cell line-derived nerve growth factor.

age, with peak GM-CSF expression level at the age of four weeks. This upregulation might be attributed to changes in hormone levels (such as testosterone and FSH) at this age. This expression of GM-CSF may directly and/or indirectly affect the functionality of testicular cells, and may also be related to the stage of development/differentiation of somatic cells, mainly Sertoli and Leydig cells, at this age. However, knockout GM-CSF mice are fertile [45], which may indicate that testicular GM-CSF is not a crucial/critical factor for the development of normal spermatogenesis and male fertility.

In order to investigate the role of GM-CSF in the process of spermatogenesis, we used the methylcellulose three-dimensional (3D) *in vitro* culture system (MCS) that was developed in our laboratory, which demonstrates, in some cases, the division and differentiation of spermatogonial cells to round spermatids, and even to the stage of sperm-like cell morphology under certain conditions [29, 30, 35-39, 46]. With this unique system, based on IF staining and qPCR analyses, we show that the addition of recombinant GM-CSF to the MCS, containing isolated cells from the seminiferous tubules of immature mice, resulted in an increase in the percentage of pre-meiotic and meiotic cells as well as an increase in the mRNA level of pre-meiotic/meiotic cell markers, but did not affect the percentage of post-meiotic cells or level of mRNA post-meiotic cell marker, compared to the control group. Our findings may indicate and suggest the possible involvement of GM-CSF in the regulation of pre-meiotic and early meiotic spermatogenesis.

This is the first study to suggest a possible involvement of GM-CSF in the development of specific stages of the spermatogenesis process in immature mice under *in vitro* conditions. The *in vitro* effect of GM-CSF on the process of spermatogenesis could be a direct effect on the pre-meiotic and meiotic cells that express GM-CSFR, and/or indirectly through the somatic cells that are present in the MCS and express GM-CSFR, such as Sertoli and Leydig cells. GM-CSF is considered a factor produced by the spermatogonial cells of the microenvironment, such as macrophages, Leydig cells, and Sertoli cells [47], and it is possible that, in response to GM-CSF, somatic cells could produce paracrine/autocrine factors that induce the development of pre-meiotic and meiotic cells, to more differentiated stages during the process of spermatogenesis. Under *in vivo* conditions, this could affect the proliferation and development of spermatogonial cells as well as Sertoli cell functions, as a paracrine factor secreted from the interstitial cells (macrophages and Leydig cells), since it can be transferred through the testicular blood barrier into the seminiferous tubule [24].

Testosterone is secreted by Leydig cells following stimulation with LH and plays an essential role in the development of normal spermatogenesis [48]. Our results show that the addition of testosterone to the MCS containing isolated cells from the seminiferous tubules of immature mice had no effect on the percentage of pre-meiotic cells. However, testosterone significantly increased the mRNA levels of pre-meiotic and meiotic cell markers. In contrast, testosterone significantly increased the percentage of meiotic and post-meiotic cells that developed in the culture but not the post-meiotic mRNA cell marker, as examined by qPCR analysis. The differences in the effect of testosterone on the level of markers of the different stages could be attributed to different mechanisms of action at the level of transcription and translation and/or to the integrity of protein or RNA of the examined markers. The findings of our study may indicate a direct involvement of testosterone in the regulation of the different stages of spermatogenesis under *in vitro* culture conditions. These results are consistent with the activity of testosterone *in vivo*, as it affects the survival and maintenance of spermatogonial cells and induces their differentiation to meiotic stages, specifically round spermatids, between stages VII and VIII in mice [19, 20].

In addition, we show that, in the presence of GM-CSF, testosterone did not affect the percentage of pre-meiotic cells, but caused a significant decrease in the mRNA level of pre-meiotic markers. Also, testosterone, in the presence of GM-CSF, did not have any significant effect on either the level of the mRNA marker or percentage of meiotic cells, compared to control. However, addition of both factors significantly increased both the mRNA marker level and percentage of post-meiotic cells in the MCS, compared to control. In addition, the effect of GM-CSF on the development of post-meiotic cells, in the presence of testosterone, was dose-dependent. These findings suggest the possibility that although GM-CSF alone does not significantly affect the meiotic/post-meiotic stages in the MCS, the presence of testosterone significantly increases (potentiate) the capacity of GM-CSF to induce the differentiation/maturation of spermatogonia cells into the post-meiotic stages. This could be related to factors produced by somatic cells in the culture in response to both GM-CSF and testosterone. Also, it is possible that the presence of testosterone and GM-CSF together lead to the maintenance and or survival of developed meiotic/post-meiotic cells. Recently, we demonstrated the involvement of

colony-stimulating factor-1 (CSF-1) and IL-34 in the development of spermatogenesis *in vitro* [42, 43]. Thus, our present and previous studies emphasize the possible involvement of factors produced by cells of the microenvironment to support spermatogonial cells in their proliferation and maturation under *in vitro* conditions.

Previous research has shown that GM-CSF increases the amount of tissue testosterone in prostatic adenocarcinoma *in vivo* [49]. In the present study, we examined the effect of GM-CSF on the expression level of 3β-HSD in the MCS; this enzyme is involved in the synthesis of testosterone by Leydig cells [50]. Our results show that GM-CSF increases the expression level of 3β-HSD *in vitro* (data not shown). Thus, the additive effect of GM-CSF and testosterone could be related to *de novo* increase in testosterone production and or sustainability over time in culture.

Lastly, our results show that testosterone increases the mRNA of ABP and GDNF but not AR, compared to control. In contrast, the addition of GM-CSF alone did not show any significant effect on the mRNA level of the examined factors produced by Sertoli cells (ABP, AR, and GDNF). On the other hand, addition of testosterone in the presence of GM-CSF significantly increased the level of AR mRNA, compared to control and GM-CSF alone. A previous study showed that testosterone triggers GDNF secretion by peritubular myoid (PM) cells *in vitro* [51]. Thus, we suggest that testosterone increases the expression of GDNF in Sertoli cells in our culture, and may also increase the expression of GDNF in residual peritubular cells present in the culture. Some studies have shown that testosterone may enhance the immunodetection of AR by decreasing the rate of degradation of AR, and another study showed that androgen increases AR protein but has no effect on AR mRNA expression in cultured Sertoli cells [52]. Also, studies have revealed that testosterone up-regulates ABP in rat Sertoli cells *in vitro* [53] and that LH induces the secretion of testosterone from Leydig cells which upregulates glycosylated ABP in Sertoli cells *in vivo* [54]. Our results are consistent with this study and moreover show an increase in the expression level of ABP in our culture following treatment with testosterone.

In the present study, we demonstrate the involvement of GM-CSF, as a paracrine/autocrine factor in the testis, in the regulation of spermatogenesis *in vitro*, and further demonstrate that testosterone potentiates GM-CSF-induced differentiation of spermatogonial cells. This may assist in enhancing the maturation of spermatogonial cells *in vitro* from prepubertal mice, and may deepen our understanding of the biomolecular interactions between somatic cells and spermatogonial cells in the testis under *in vivo* conditions that lead to complete spermatogenesis. Furthermore, these results may lead to the development of future therapeutic strategies regarding the preservation of prepubertal male fertility.

DISCLOSURE

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