

Interleukin-6 expression during normal maturation of the mouse testis

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ABSTRACT. In this study, we examined the cellular origin and the expression levels of interleukin-6 (IL-6) during normal maturation of mouse testis. The levels of IL-6 (protein and mRNA) were higher in testicular homogenates of sexually immature than mature mice. Immunohistochemical staining of testicular tissues of sexually immature and adult mice show that testicular germ cells, at different stages of differentiation, Leydig cells/interstitial cells and peritubular cells express IL-6. Our results demonstrate, for the first time, overexpression of IL-6 in testicular tissues of immature mice, as compared to mature mice, as well as the expression of IL-6 in germ cells of testicular tissues of adult and sexually immature mice. Thus, our results may indicate the involvement of the endocrine system (gonadotropins and testosterone) in the regulation of IL-6, which is involved in the regulation of testicular development, functions and spermatogenesis.

Keywords: interleukin-6, Sertoli cells, Leydig cells, germ cells, testis, LPS

Interleukin-6 (IL-6) is a multifunctional cytokine, produced mainly by macrophages in response to foreign antigens, pathogens (infection challenge) and also in chronic inflammation (immunologic activation) [1-4]. Cells of non-immune cell origin, such as dendritic cells, fibroblasts, endothelial cells, smooth muscle cells, mesangial cells, astrocytes and epithelial cells, also produce IL-6 [1-4].

IL-6 acts through the gp 130 receptor, and affects proliferation or differentiation and the functioning of B cells, T cells, hepatocytes, hematopoietic progenitor cells, neuronal cells and trophoblasts [1-4]. In addition to its function as a pro-inflammatory cytokine, IL-6 also functions as anti-inflammatory cytokine by inducing the production of interleukin-1 receptor antagonist (IL-1ra), soluble tumor necrosis factor alpha (sTNF- α) and decreasing the production of TNF [3, 4].

In mammals, the process of spermatogenesis occurs within the seminiferous tubules that release spermatozoa into the rete testis. The seminiferous tubules contain germ cells and Sertoli cells. Peritubular meyooid cells (PC) surround the tubules and are in contact with the basal surface of the Sertoli cells and spermatogonia. Leydig cells are located in the interstitium of the testis between tubules [5].

Spermatogenesis is a process, which is highly controlled by endocrine and paracrine/autocrine factors that affect the testicular cell-cell interactions [6, 7]. These factors control

proliferation, meiosis, and differentiation, which occur in a variable number of sets of spermatogenic cell associations or stages [5-8]. There are probably a number of cytokines that are involved in the regulation of various differentiation steps in this process [9, 10].

Cytokines, including IL-6, have been demonstrated in the seminal plasma of fertile and infertile men [11]. Under *in vitro* condition, it was demonstrated that Sertoli cells secrete IL-6, which was increased following stimulation using residual bodies (organized from late spermatids at the time of spermiation), with low levels of testosterone and FSH [12, 13]. The levels of IL-6 varied throughout the different stages of the seminiferous epithelium cycle; high levels were observed in stages II-VI and the lowest levels in stages VII-VIII [12]. Leydig cells also express IL-6 following stimulation with LH *in vitro* [14]. There is no information about the cellular localization of the IL-6 receptor in the testicular cells. IL-6 has been suggested to be a potent inhibitor of meiotic DNA synthesis (mostly preleptene spermatocytes and to lesser degree in spermatogonia), within the seminiferous epithelium and a stimulator of transferrin production by Sertoli cells [15, 16].

In the present study, we examined the cellular origin and the expression levels of IL-6 during normal maturation of mouse testis.

MATERIALS AND METHODS

Materials

Interleukin-6 levels in testicular homogenates were measured using a murine IL-6 Eli-pair kit (Diaclone, Besançon, France). This kit recognizes both natural and recombinant murine IL-6, and has no cross-reactivity with other murine cytokines. The range of the standard curve was 2 to 500 pg/mL, and the sensitivity of the kit was < 32 pg/mL.

Casein, proteinase K, Tween 20, diamino-benzidine tetrahydrochloride (DAB) were purchased from Sigma (MO, St. Louis, USA); Urea (Analar, BDH). Biotinylated antibodies, streptavidin-peroxidase conjugate and normal goat serum, from Zymed (San Francisco, CA, USA), Eukitt (GmbH). All other chemicals (analytical grade) were purchased from commercial sources.

The investigations were conducted in accordance with the Guiding Principles for the Care and Use of Research Animals promulgated by the Society for the Study of Reproduction. Sexually mature (adults) (8-10 weeks old) and sexually immature (2 weeks old) Balb/c mice were used. At the age of 2 weeks, even though Sertoli cells are almost completely differentiated, the spermatogenic process is not complete and mice are not producing spermatozoa [17]. Three mice were examined in each experiment and each experiment was repeated more than 6 times.

Preparation of testicular homogenates

Testicular homogenates were prepared from immature and mature mice. A single testis from each mature mouse and two testes from each immature mouse were prepared and examined separately. The tunica albuginea was removed from the testis and the remaining testicular tissue was homogenized in 0.8 mL cold PBS over ice. At the end of the homogenization process, the mixture was centrifuged at 13 000 RPM for 15 min and the supernatant was collected and stored at -70 °C. Total protein was investigated using Biorad reagent. IL-6 levels were measured using a specific ELISA kit.

Immunohistochemical staining of mouse testicular tissues

Four-micron-thick sections from formalin-fixed, paraffin-embedded testicular tissue blocks of adult and immature mice were mounted on saline-coated slides, dried at 37 °C for 48hr and stored at room temperature. Before the primary antibodies were applied, blocking of the nonspecific background was done with PBS containing 0.05% casein and/or normal goat serum. This solution was also used to dilute the primary antibodies. Sections were boiled in 6 M urea for 10 min [18]. Thereafter, polyclonal rabbit anti-mouse IL-6 antibodies (10 µg/mL) were used as primary antibodies. After the primary antibodies had been applied for 1 hour, the PBS/casein solution was used for all further washings. The biotinylated antibody and the streptavidin-peroxidase conjugate were applied according to the suppliers' directions. Endogenous peroxidase was blocked with 3% H₂O₂ in 80% methanol for 15 min. before the streptavidin-peroxidase conjugate was applied. Development was performed with 0.06% DAB, and Mayer's haematoxylin was used for counterstaining. The sections were

mounted in Eukitt. Negative controls were included for each specimen using PBS/casein instead of the primary antibodies and/or by pre-absorption of the first anti-IL-6 antibodies with the recombinant IL-6 which showed a significant decrease as compared with the positive staining (*figure 1D*).

Pre-absorption of the primary antibodies

Antibodies (anti-IL-6, 10 µg/mL) were incubated with various concentrations (1-30 µg/mL) of recombinant IL-6. After overnight incubation at 4 °C, the mixture was used as the primary antibody to stain the testicular tissues. An example is depicted in *figure 2D* for IL-6 staining after pre-absorption.

Extraction of total RNA and RT-PCR analysis

Total RNA was extracted from mouse testis using the EZ RNA Reagent protocol (Biological Industries, Beit Haemek, Israel). First-strand complementary DNAs (cDNAs) were synthesized from 2.5 µg total RNA with 0.5 µg random oligonucleotide primers (Roche Molecular Biochemicals, Mannheim, Germany) and 200 U of Moloney-Murine Leukemia Virus-Reverse Transcriptase (M-MLV-RT; Life Technologies, Inc., Paisley, Scotland, UK) in a total volume of 20 µL Tris-HCl-MgCl reaction buffer, supplemented with DTT, dNTPs (0.5 mmol/L; Roche Molecular Biochemicals) and RNase inhibitor (40 U; Roche Molecular Biochemicals). The reverse transcriptase (RT) reaction was performed for 1 h at 37 °C and stopped for 10 min at 75 °C. The volume of 20 µL was subsequently made up to 60 µL with water. Negative controls for the reverse transcriptase reaction (RT-) were prepared in parallel, using the same reaction preparations with the same samples, without M-MLV-RT.

The PCR performed subsequently, contained cDNA samples in a final dilution of 1:15, with two pairs of oligonucleotide primers (0.9 pmol/µL; 5'AGAGG-GAAATCGTGCGTGAC3'; and 3'GCCGGACTCATCG-TACTCT5' for the mouse β-Actin cDNA sequence, and 5'GACGATACCACTCCCAACAGACC3;' and 3'AT-GCTTAGGCATAACGCACTAGGTT5' for the mouse IL-6 cDNA sequence (Sigma). To assess the absence of genomic DNA contamination in the RNA preparations and RT-PCR reactions, PCR was performed with the negative controls of the RT reaction (RT-) and without cDNA (cDNA-). The PCR reactions were carried out on a Cyler II System Thermal Cyler (Ericomp, San Diego, CA, USA). Twenty microliters of each PCR product were run on 2% agarose gel, containing ethidium bromide, and photographed under UV light.

The RNA expression was quantified from the different samples of the RT-PCR using TINA software (version 2.10g) (raytest Isotopenmessgeraets, GmbH, Straubhardt, Germany).

Evaluation of results

Each experiment included three to five adult mice and three to five immature mice. Each experiment was repeated at least three to six times. Immunohistochemical staining was calculated by intensity, from 0 to 3, which included intensity of the staining,

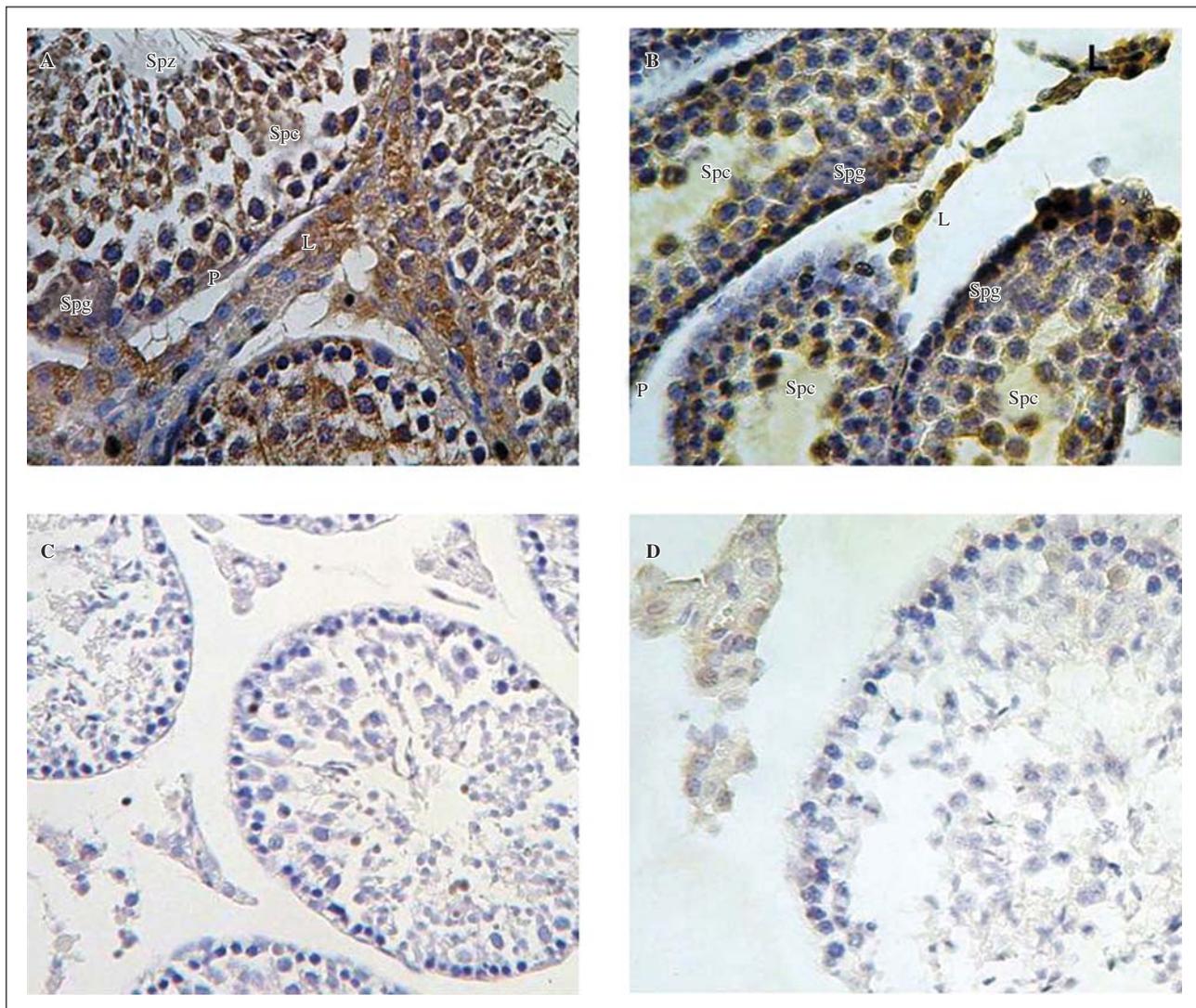


Figure 1

Expression of IL-6 in testicular tissues of adult and sexually immature mice.

Immunohistochemical staining of testicular tissues from adult (A) and sexually immature mice (B) with polyclonal rabbit anti-mouse IL-6 antibodies (10 $\mu\text{g}/\text{mL}$) (x 400). As negative control (C), we stained tissues with secondary antibodies alone, or the primary anti-IL-6 antibodies were pre-absorbed with recombinant IL-6 (D).

P: peritubular cells; L: Leydig cells (interstitial cells); Spg: spermatogonia; Spc: spermatocytes; Spz: spermatozoa.

and percentage of the stained cells in a 10 high power field (HPF).

The levels of IL-6 were evaluated as $\text{pg}/\mu\text{g}$ protein of the testicular homogenate. The results are presented as the mean of $\text{pg}/\mu\text{g}$ protein \pm SEM.

Statistics

Student's *t* test was used for statistical evaluation, and *p* values below 0.05 were considered significant.

RESULTS

Expression of IL-6 in testicular tissues of adult and sexually immature mice

Immunohistochemical staining of formalin-fixed, paraffin-embedded testicular tissues of adult mice show that spermatogonia, spermatocytes and interstitial cells (which are composed mainly of Leydig cells) express IL-6

(figure 1A). In addition, the tails of the spermatozoa were also positively stained for IL-6. Peritubular cells also express IL-6. The same pattern of IL-6 expression was found in testicular tissue cells from sexually immature mice (figure 1B). The negative control, using only the secondary antibody (figure 1C), did not show IL-6 expression, and/or using pre-absorbed, primary anti-IL-6 antibodies with rIL-6, showed only low, non-specific staining of IL-6 (figure 1D).

Overexpression of IL-6 in testicular homogenates of immature, as compared to mature, mice

As depicted in figure 2, testicular homogenates from immature mice contain significantly higher levels of IL-6 as compared to mature mice (0.119 ± 0.04 and 0.033 ± 0.01 $\text{pg}/\mu\text{g}$ protein respectively; $p = 0.0000002$). IL-6 mRNA expression in testicular tissues from immature mice was significantly higher than in testicular tissues

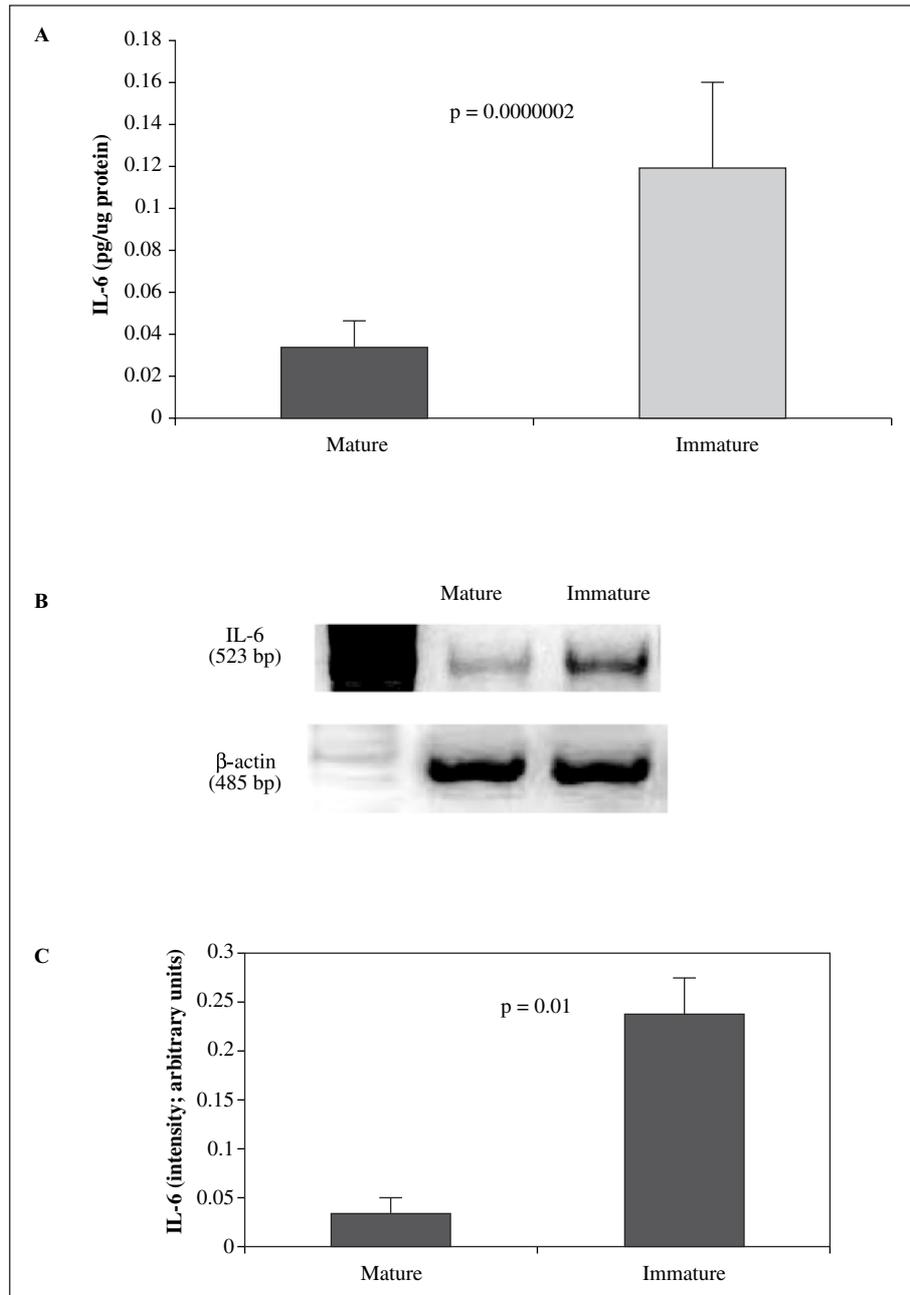


Figure 2

Overexpression of IL-6 in testicular tissues of immature as compared to mature mice.

IL-6 levels were examined in homogenates of testes from 25 adult and 16 immature mice. IL-6 protein levels were evaluated by a specific ELISA kit. Levels are expressed as pg/ μ g protein \pm SEM (A). The expression levels of IL-6 mRNA were evaluated by RT-PCR using specific primer, and IL-6 PCR products were separated on 2% agarose gel containing ethidium bromide (B). Quantitative evaluations of the PCR products were performed using video densitometry, and the ratio between IL-6 bands and β -actin bands were calculated (C). The results are expressed as arbitrary units.

from immature mice as evaluated by RT-PCR (figure 2B) and quantified by densitometry ($p = 0.01$) (figure 2C).

DISCUSSION

The involvement of all testicular cells in the production of IL-6 suggests that IL-6 could be involved in the regulation of crucial physiological functions in the testis, such as development, growth, proliferation and differentiation. The higher levels of IL-6 found in the testis of sexually immature mice than those found in the testis of mature

mice may indicate that endocrine factors could regulate its expression. LH has been shown to regulate the proliferation of Leydig cells [19], and IL-6 levels have been shown to be increased following stimulation of Sertoli cells with FSH [12].

Also, IL-6 was secreted by mature spermatozoa [20]. Thus, IL-6 could be considered to be a paracrine signal between Sertoli cells and spermatid/spermatozoa cells within the seminiferous tubules.

Several experimental models suggest that IL-6 is a protective factor against inflammation, by inducing anti-inflammatory factors such as IL-1ra, sTNFR I, and even by

decreasing TNF- α production [4]. Thus, IL-6 could play a role in testicular function under both physiological and pathological conditions.

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