Over-expression of IL-18, ICE and IL-18 R in testicular tissue from sexually immature as compared to mature mice

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ABSTRACT. In this study we examined the cellular origin and the expression levels of interleukin-18 (IL-18), IL-18 receptor (IL-18R) and IL-1β-converting enzyme (ICE), which activates pro-IL-18, during normal maturation of murine testis. The levels of IL-18, IL-18R and ICE were significantly higher in testicular tissues and homogenates (but not in the spleen or liver) from sexually immature than mature mice. Immunohistochemical staining of testicular tissues from sexually immature and mature mice shows that testicular germ cells and Leydig cells/interstitial cells express higher levels of IL-18, as compared to other testicular cells. Peritubular cells of sexually immature and mature mice also expressed IL-18. Our results demonstrate, for the first time, over-expression of the IL-18 family in testicular tissues of sexually immature mice, as compared to mature mice, as well as the expression of IL-18 in the different stages of differentiation of testicular germ cells. Thus, our results may indicate involvement of the endocrine system (gonadotropins and testosterone) in the regulation of the testicular IL-18 family, which could be involved in the regulation of testicular functions, development and spermatogenesis under physiological conditions.

Keywords: interleukin-18, ICE, testis, development, spermatogenesis

In mammals, the process of spermatogenesis occurs within the seminiferous tubules that release spermatozoa into the rete testis. The seminiferous tubules contain germ cells (GC) and Sertoli cells (SC). Peritubular myoid cells (PC) surround the tubules and are in contact with the basal surface of the Sertoli cells and spermatogonia. Leydig cells (LC) are located in the interstitium of the testis between tubules [1].

Spermatogenesis is a process of proliferation and differentiation of germ cells, and is under the control of endocrine and autocrine/paracrine factors [1, 2]. In addition to the regulatory effect of gonadotropins and androgens in the initiation and maintenance of spermatogenesis, a number of cytokines are also involved in the regulation of various differentiation steps in this process [3-5].

Cytokines, such as the IL-1 family, IL-6 and TNF-α, have been detected in lysates and cells of the testicular tissue and also in seminal plasma of fertile and infertile men [6-15].

IL-18 is a multifunctional pro-inflammatory cytokine, which was formerly called interferon-γ (IFN-γ)-inducing factor, and is a potent inducer of IFN-γ production. It participates in the regulation of both T helper cells type 1 (Th1) and Th2 responses, and also in that of innate and acquired immunity [16, 17]. IL-18 plays a key role in host defense against various infectious agents by inducing the secretion of various cytokines, FasL and nitric oxide (NO) and enhancement of natural killer cell (NK) cytotoxicity [18-21]. Initially detected in the serum of animals with endotoxemia, it is produced by a wide variety of cells such as monocytes, macrophages, keratinocytes, osteoblasts, intestinal epithelial cells, microglial cells, as well as cells from the adrenal cortex [18-21]. IL-18 belongs to the IL-1 family of ligands. Like IL-1β, IL-18 is a cytoplasmic protein synthesized as a biologically inactive 24-kDa precursor molecule lacking a signal peptide that requires cleavage into an active, mature 18 kDa molecule by the intracellular cysteine protease called IL-1β-converting enzyme (ICE or caspase-1) [18, 22, 23]. The IL-18 receptors, although distinct from IL-1 receptors, also belong to the IL-1 family. IL-18 receptor (IL-18R) complex is a heterodimer, consisting of a ligand binding chain (IL-18Rα) and a co-receptor chain (IL-18Rβ); both chains are required for signaling [24, 25].

Since IL-18 was recently demonstrated in rat and mouse testis [26, 27], and it shares similar functions with IL-1β [17, 28-33], in the present study we examined the cellular origin and the expression levels of IL-8, IL-18R and ICE during normal maturation of murine testis.

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METHODS AND MATERIALS

Reagents

Interleukin-18 levels in testicular homogenates were measured using a murine IL-18 ELISA kit. Lyophilized recombinant mouse IL-18 (Code No. B002-5), monoclonal rat anti- mouse-IL-18 antibodies (Code No. D047-3, clone 74) and biotinylated rat anti-mouse IL-18 antibodies (Code No. D048-6, clone 93-10c) for ELISA, were purchased from MBL (Medical & Biological Laboratories Co., LTD, Naka-ku, Nagoya, Japan, specific for mouse only). Rabbit polyclonal anti-mouse IL-18 antibodies (H-173, sc-7954) (cross reactive with mouse, rat and human) were purchased from Santa Cruz Biotechnology; Inc. (CA, USA). The range of the standard curve was 2-500 pg/mL, and the sensitivity of the kit was < 16 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 (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 (two weeks old) BALB/c mice (Harlan Laboratories, Jerusalem, Israel) were used. At the age of two weeks, although Sertoli cells are almost completely differentiated, the spermatogenic process is incomplete and mice are not producing spermatozoa [34].

Preparation of testicular homogenates

Testicular homogenates were prepared from mature mice. A single testis from each mature mouse was prepared and examined separately. The tunica albugina was removed and the remaining testicular tissue was homogenized in 0.8 mL cold PBS in ice. At the end of the homogenization process, the mixture was centrifuged at 13 000 RPM for 15 min, the supernatant was collected and stored at -70°C. Total protein was examined by Biorad reagent according to the manufacture's instructions (BIO-RAD Laboratories GmbH, Munchen, Germany). The Bio-Rad Protein Assay is a dye-binding assay based on the differential color change of a dye in response to various concentrations of protein [35].

Extraction of total RNA, reverse transcriptase (RT)-PCR and real-time PCR analysis

Total RNA was extracted from murine testis, spleen and liver 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 but without M-MLV-RT.

RT-PCR analysis

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′AGAGGGAAATCGTGCGTGAC3′; and 3′GCCGGACTCATCGTACTCCT5′ for the murine β-Actin cDNA sequence, 5′AAGGCGCATGTGTGCTAATC3;' and 3′ TGTACAACCGCAGTAATACGGAAT5′ for the murine IL-18 cDNA sequence; 5'ATTTAAGGTCCAATTGCGACGA3' and 3'GTGCACAGGAATGAAACAGC5' for the murine IL-18Rα cDNA sequence; and 5'TGGTGTTGAAGAGCAGAAAGC3' and 3'CAGAGCACAAGACTTCTGAC5' for the murine ICE cDNA sequence (Sigma). To assess the absence of genomic DNA contamination in RNA preparations and RT-PCR reactions, PCR was performed with negative controls of the RT reaction (RT-) and without cDNA (cDNA-). The PCR reactions were carried out on a Cycler II System Thermal Cycler (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.

For ICE we used 35 cycles, for IL-18R we used 40 cycles, and for IL-18 we used 30 cycles.

The IL-18 protein levels were quantified from the different samples of the western blot using TINA software (version 2.10 g) (raytest Isotopenmessgeraets, GmbH, Straubenhardt, Germany).

Real-time PCR analysis

Real-time, quantitative, PCR amplification of total cDNA (500 ng/sample) used specific primers of the IL-18 family sequences as follows: IL-18 forward primer, 5′-CAGGCCTGACATCTTCTGCAA-3′; reverse primer, 5′-CTGACATGGCAGCCATTGT-3′, ICE forward primer, 5′-CACTGGCAGGAATTCTGGA-3′; reverse primer, 5′-ATGATCACCTTGGGCTTGTC-3′. IL-18R forward primer, 5′-TTAGGACCAAAGTGTGAGAAGG-3′; reverse primer, 5′-TCTCGTCTCTTTCCGCTATGCG-3′. β-actin forward, 5′-AGAGGGAAATCGTGCGTGAC-3′; reverse primer, 5′-CAATAGTGACCTGGCCGT-3′ (Sigma). The reactions were conducted following the protocol for the Absolute qPCR SYBR Green mix (ABgene House, Blenheim Road, Epsom, UK), containing modified Tbr DNA polymerase, SYBR Green, optimized PCR buffer, 5mM $MgCl_2$, dNTP mix, and dUTP. The PCR reaction was performed using a real-time PCR machine (MyIQ, Bio-Rad Laboratories, USA) according to the manufacturer's instructions. The following PCR protocol, repeated 45-50 times, was used: denaturation (95°C for 10 min), amplification and quantification (94°C for 10 s, 60°C (IL-18, ICE, IL-18R and β-actin), 72°C for 30 s with a single fluorescence measurement, melting curve (60-95°C with a heating rate of 0.5°C per 30 s and a continuous

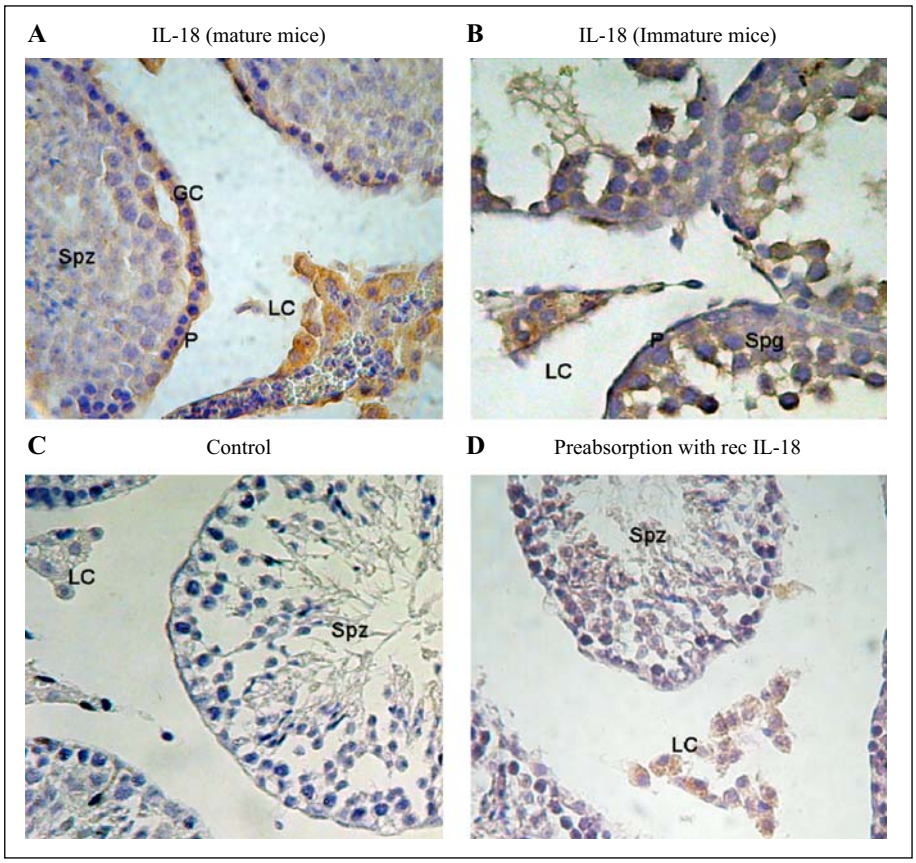

Figure 1

Localization of IL-18 in testicular cells from mature and sexually immature mice. Immunohistochemical staining of testes from sexually mature (**A**) and sexually immature mice (**B**) with polyclonal rabbit anti-mouse IL-18 antibodies (10 µg/mL) (x 400) were performed. As negative control, testicular tissues were stained with second antibodies and normal serum alone (**C**), or with the first anti-IL-18 antibodies which were pre-absorbed with recombinant IL-18 (**D**).

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

fluorescence measurement), and a cooling step to 4°C. PCR products were identified and distinguished by the melting curve generated. The "threshold cycle" (Ct) values, representing the cycle number at which sample fluorescence rose statistically above background and crossing points (CP) for each transcript, were defined. The relative quantity of gene expression was analyzed by the 2-ΔΔCt method. The quantities of IL-18, ICE and IL-18R mRNA were normalized to the endogenous control, β-actin.

To ensure accurate identification of product and the absence of contaminating DNA, all products of the RT-PCR which were used for real time PCR, for all the factors examined, were also examined in parallel by PCR analysis with all the negative controls, and were found to give a single specific band with the suitable size when run on 2% agarose gel.

Immunohistochemical staining of murine testicular tissues

Four-micron-thick sections from formalin-fixed, paraffin-embedded, testicular tissue blocks of adult and sexually immature mice were mounted on saline-coated slides, dried at 37°C for 48 hrs 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 6M urea for 10 min [36]. Thereafter, polyclonal rabbit anti-mouse IL-18 antibodies (10 µg/mL) were used as primary antibodies. After the primary antibodies had been applied for 1 hour, a 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% H_2O_2 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 counter staining. The sections were mounted in Eukitt. Negative controls were included for each specimen using normal serum instead of the primary antibodies and/or by pre-absorption of the first anti-IL-18 antibodies with the recombinant IL-18, which showed a significant decrease as compared with the positive staining *(figure 1D)*.

Pre-absorption of the first antibodies

Anti-IL-18 (10 µg/mL) was incubated with various concentrations (1-30 µg/mL) of recombinant IL-18. After overnight incubation at 4°C, the mixture was used as the first antibody to stain the testicular tissues. An example is depicted in *figure 1*D for IL-18 staining after pre-absorption.

Western blot analysis

Testicular homogenates were incubated in sample buffer [0.02% Bromophenol blue and 2% Dithiothreitol (DTT)]. Aliquots (50 µg protein) from the samples were submitted to electrophoresis on a 12% sodium dodecylsulfate (SDS)-polyacrylamide gel. The separated peptides were transferred onto nitrocellulose membranes, and the latter were blocked by incubation with milk at 25°C for 2 hour. The blots were sequentially incubated for 12 hours at 4°C with milk containing primary antibody [polyclonal rabbit anti-murine IL-18 (0.2 µg/mL) l)], [polyclonal rabbit anti-murine ICE (0.4 µg/mL) l)], monoclonal anti-human IL-18Rα antibodies (1 µg/mL) (Cat. No. MAB840, clone 70625; R&D Systems, Inc. Minneapolis, MN, USA) or murine monoclonal anti-murine β-actin (0.6 µg/mL) (Sigma, Saint Louis, MI, USA). After three wash cycles with PBS-Tween 20, secondary antibodies (diluted in milk) were added to the nitrocellulose filters and developed in Enhanced Chemiluminescent (ECL) (Biological Industries, Beit Haemek, Israel).

Evaluation of results

Each experiment included three to five adult mice and three to five sexually immature mice and was repeated at least three to six times.
The levels of IL-18 were evaluated as pg/µg protein of the testicular homogenate. The results are presented as mean of pg/µg protein ± SEM.

Statistics

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

RESULTS

Expression of IL-18 in testicular tissues from sexually immature and mature mice

Immunohistochemical staining of formalin-fixed, paraffin-embedded testicular tissues of sexually mature mice show that spermatogonia and interstitial cells (which are composed mainly of Leydig cells and macrophages) express higher levels of IL-18 as compared to other testicular cells, such as peritubular cells, differentiated germ cells and Sertoli cells *(figure 1A)*. The same pattern of IL-18 expression was found in testicular tissue cells from sexually immature mice *(figure 1B)*. The negative control using only the second antibody and normal serum *(figure 1C)*, did not show IL-18 expression, and/or using pre-absorbed first anti-IL-18 antibodies with rIL-18 showed low, non-specific staining of IL-18 *(figure 1D)*.

Over-expression levels of IL-18 in testicular homogenates from sexually immature as compared to mature mice

As depicted in *figure 2A*, testicular homogenates from sexually immature mice contain significantly higher levels of IL-18 as compared to mature mice (0.11 ± 0.06 and 0.03 ± 0.01 pg/µg protein respectively; $p < 0.0001$), as examined by specific ELISA.

Testicular homogenates from sexually immature and mature mice contain a 24 kDa IL-18 peptide (the inactive form, according to the molecular weight) and an 18 kDa IL-18 peptide (the active form, according to the molecular weight) *(figure 2B)*. The levels of the 24 kDa and 18 kDa forms were significantly higher in the testicular homogenates from sexually immature as compared to mature mice ($p < 0.01$ and $p < 0.001$, respectively), as identified by western blot analysis (*figure 2B*; a representative experiment is presented) and quantified by densitometry (*figure 2C*; a summary of three different and independent experiments).
In addition, the expression levels of IL-18 mRNA were significantly higher in testicular tissues from sexually immature as compared to mature mice, as evaluated by real time PCR analysis ($p < 0.00001$) *(figure 2D)*.

Over-expression of ICE levels in testicular homogenates from sexually immature as compared to mature mice

The enzyme ICE that processes pro-IL-18 (inactive form) to its mature (active) form, which is synthesized as a 45-kDa polypeptide precursor (inactive form), and then transformed into an active subunit of 25 kDa, was detected in murine testicular homogenates, as examined by western blot analysis *(figure 3A, B)*. The levels of both the 45 kDa and 25 kDa ICE peptides were significantly higher in the testicular homogenates from sexually immature as compared to mature mice ($p < 0.001$), as demonstrated by western blot analysis (*figure 3A*; a representative experiment is presented), and quantified by densitometry (*figure 3B*; a summary of three different and independent experiments). Also, the expression levels of ICE mRNA *(figure 3C)* were significantly higher in testicular tissues of sexually immature as compared to mature mice ($p < 0.001$), as evaluated by real time PCR analysis.

Over-expression of IL-18R levels in testicular homogenates from sexually immature as compared to mature mice

IL-18 receptor was expressed in testicular homogenates, and the three different forms of the peptide were detected (50-75 kDa, 37 kDa and 25 kDa), as examined by western blot analysis *(figure 4A)*. The levels of both 50-70 kDa and 25 kDa IL-18R peptides were significantly higher in the testicular homogenates from sexually immature as compared to mature mice ($p < 0.001$ and 0.01, respectively), as demonstrated by western blot analysis (*figure 4A*; a representative experiment is presented) and quantified by densitometry (*figure 4B*; a summary of three different and independent experiments). Also, the expression levels of IL-18R mRNA *(figure 4C)* were significantly higher in testicular tissues fromsexually immature as compared to mature mice ($p < 0.001$), as evaluated by real time PCR analysis.

Expression levels of IL-18, ICE and IL-18Rα in spleen and liver tissues from sexually immature as compared to mature mice

The expression levels of IL-18 mRNA *(figure 5A)*, ICE *(figure 5B)* and IL-18Rα *(figure 5C)* were similar in spleen

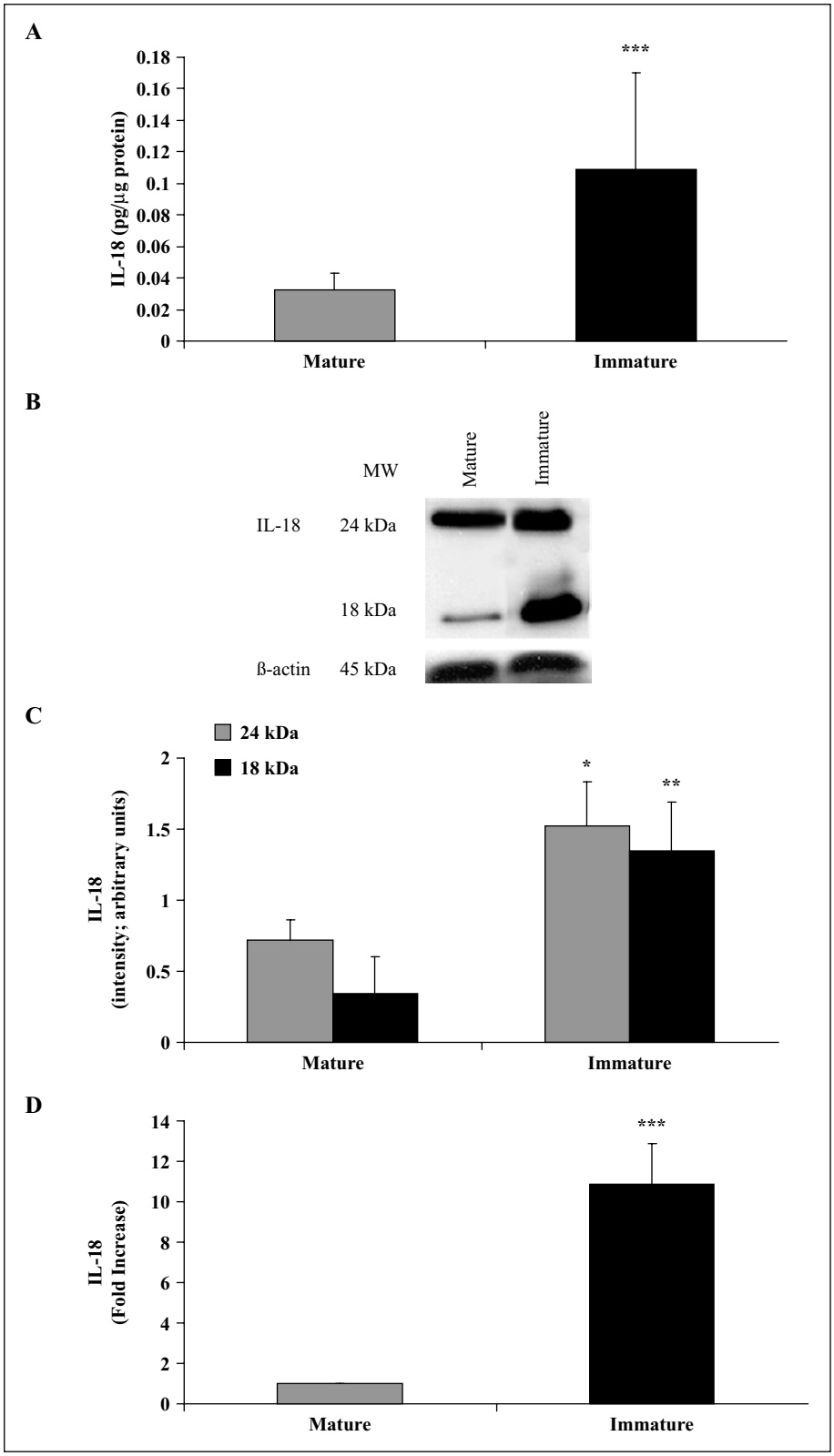

Figure 2

Over-expression levels of IL-18 in testicular homogenates from sexually immature as compared to mature mice. Testes from sexually immature (n = 23) and mature (n = 23) mice were collected and homogenized. IL-18 protein levels were evaluated by a specific ELISA kit. IL-18 levels are expressed as pg/μg protein ± SEM (**A**). IL-18 protein levels and molecular weight were evaluated by western blot analysis (**B**; a representative experiment is presented). Quantitative evaluation of the western products was performed by video densitometry (**C**; a summary of three different and independent experiments; three mice were used in each experiment). The expression levels of IL-18 mRNA were evaluated by real time PCR using specific primer (**D**).

* Indicates statistical significant between sexually immature and mature mice; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

MW: molecular weight (kDa).

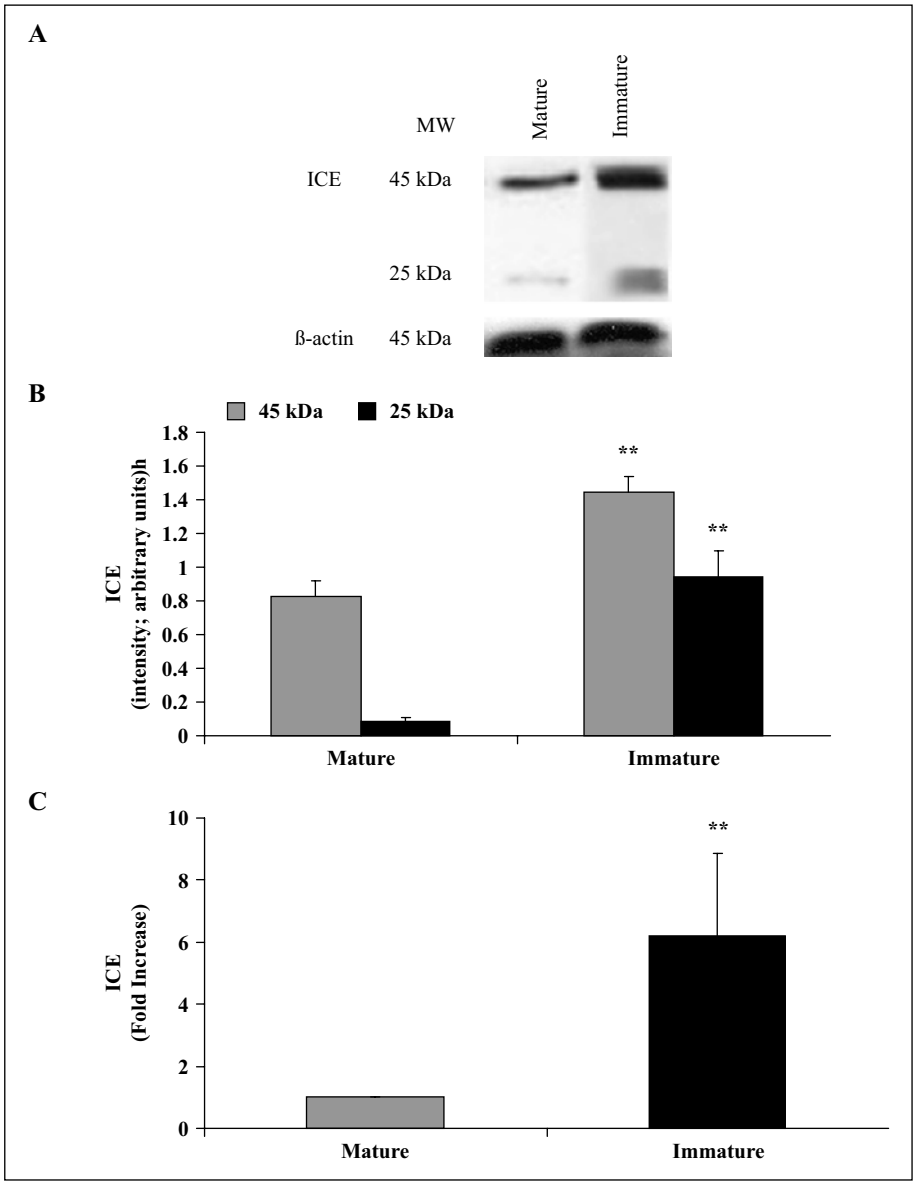

Figure 3

Over-expression levels of ICE in testicular homogenates from sexually immature as compared to mature mice. Testes from sexually immature and mature mice were collected and homogenized as mentioned in *figure 2*. ICE protein levels and molecular weight were evaluated by western blot analysis (**A**; a representative experiment is presented). Quantitative evaluation of the western products was performed by video densitometry (**B**; a summary of three different and independent experiments; three mice were used in each experiment). The expression levels of ICE mRNA were evaluated by real time PCR using specific primer (**C**).

* Indicates statistical significant between sexually immature and mature mice; ** $p < 0.01$.

MW: molecular weight (kDa).

and liver tissues from sexually immature as compared to mature mice, as evaluated by real time PCR analysis.

DISCUSSION

Using immunohistochemical studies, we were able to show that IL-18 is expressed in testicular germ cells and other tubular cells, and interstitial cells of both sexually immature and mature mice. High levels of IL-18 were expressed in testicular germ cells and interstitial cells (Leydig cells and macrophages). These results may suggest that the testicular cells are producers of IL-18, and may indicate the possibility of an autocrine/paracrine role for IL-18 in the regulation of testicular functions under physiological conditions. On the other hand, we demonstrated higher levels of IL-18, ICE and IL-18R in testicular tissues and homogenates, but not in the spleen or liver, from sexually immature than mature mice. These results may indicate the testes as specific organs where the IL-18 family, in addition to other cytokines previously demonstrated [10, 12], is over-expressed in immature as compared mature mice. Thus, it is possible to suggest that the over-expression of the cytokines examined in immature as compared to mature mice is not a general "inflammatory-like" phenomenon occurring in most tissues of the developing mouse. IL-18 family members are expressed in the testes with similar molecular weights to those which are expressed in other tissues and cells [17-25]. The present study shows that murine testicular tissues express all mem-

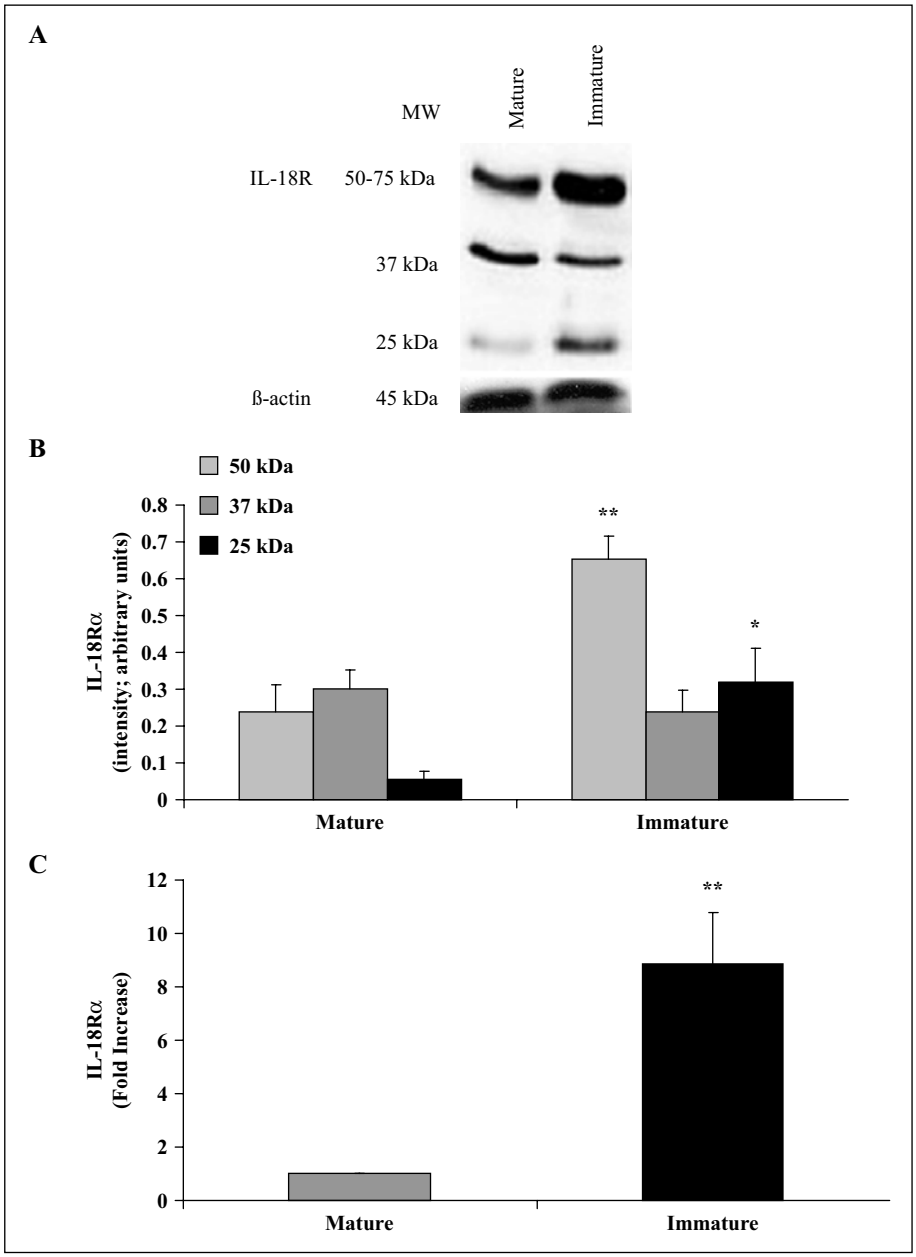

Figure 4

Over-expression levels of IL-18R in testicular homogenates from sexually immature as compared to mature mice. Testes from sexually immature and mature mice were collected and homogenized as mentioned in *figure 2*. IL-18R protein levels and molecular weights were evaluated by western blot analysis (**A**; a representative experiment is presented). Quantitative evaluation of the western products was performed by video densitometry (**B**; a summary of three different and independent experiments; three mice were used in each experiment). The expression levels of ICE mRNA were evaluated by real time PCR using specific primer (**C**).

* Indicates statistical significant between sexually immature and mature mice; * $p < 0.05$; ** $p < 0.01$.

MW: molecular weight (kDa).

bers of the IL-18 family (we also demonstrated the expression of IL-18 binding protein in testicular tissues; data not shown), which suggests that IL-18 may be involved in the regulation of crucial physiological functions in the testis, such as development, growth, proliferation and differentiation of the different cells. In addition, the changes in the levels of the IL-18 family during sexual development may indicate their different effects/functions during these periods. These results may also indicate a possible involvement of gonadotropins and testosterone in the regulation of testicular IL-18-family expression. Thus, IL-18 could be involved in the regulation of testicular cell functions, during sexual maturation, and may control spermatogenesis. Indeed, recently it was demonstrated that IL-18 stimulated DNA synthesis of germ cells during the spermatogenic stage I in rat [26].

On the other hand, IL-18 is not considered a limiting factor in testicular development and fertility, since IL-18-deficient mice were born at the expected Mendelian ratio and were phenotypically normal and fertile [37]. The differences in the extent of increase of the testicular IL-18 family between western blot and real time PCR analysis in sexually immature and mature mice could be related to regulatory mechanisms at the levels of transcription and translation.

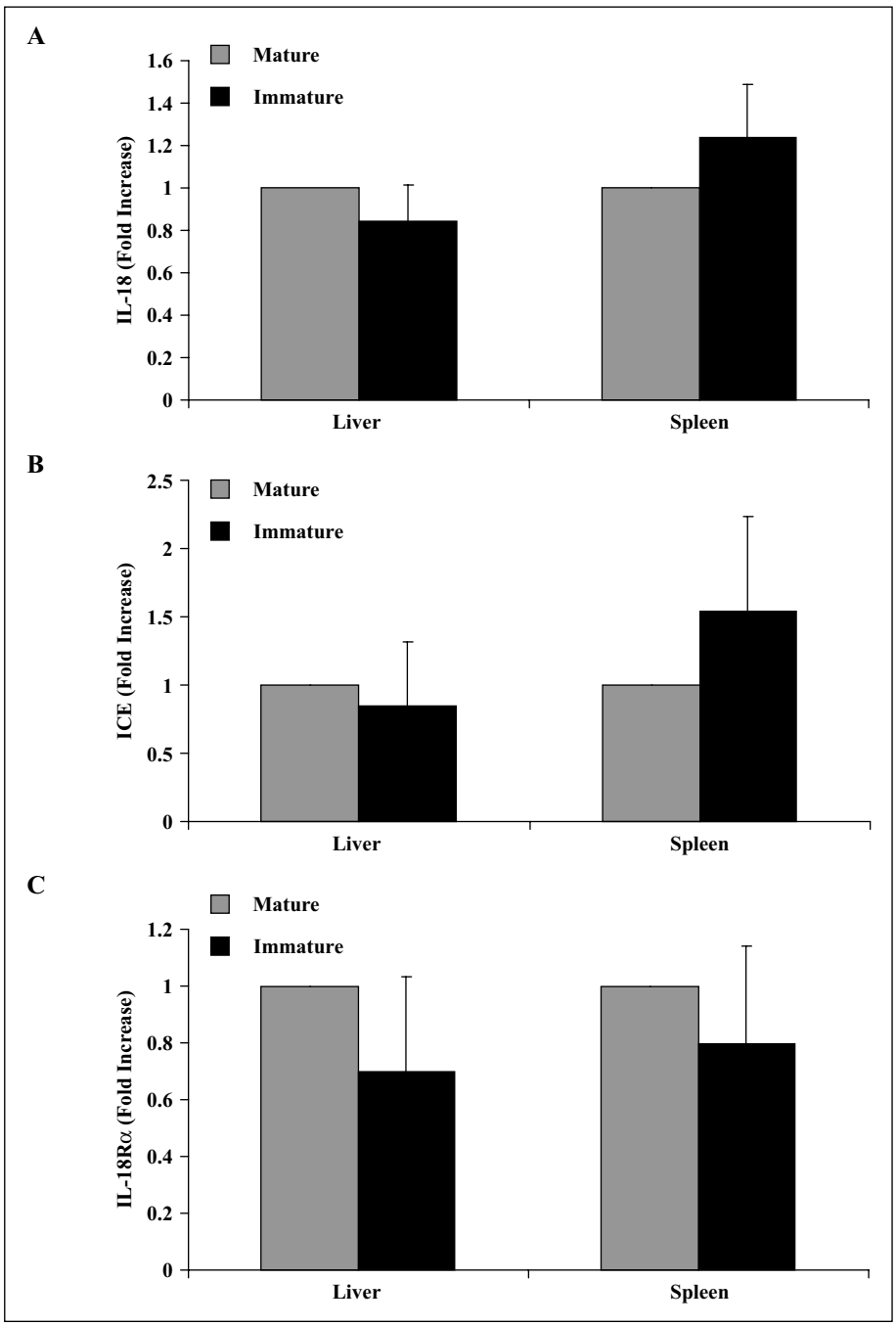

Figure 5

Expression levels of IL-18, ICE and IL-18Rα in spleen and liver tissues from sexually immature as compared to mature mice. Spleen and liver from sexually immature (n = 6) and mature (n = 6) mice were collected and individually homogenized; RNA was extracted as mentioned in *figure 2*. IL-18 (**A**), ICE (**B**) and IL-18Rα (**C**) mRNA levels were evaluated by real time PCR using specific primer.

Our results show that mouse testicular tissue produces the inactive and active forms of IL-18 (24 kDa and 18 KDa, respectively; according to the molecular weight). Our study is in contrast to a recent report by Strand *et al*., 2005 [26], who showed that rat testis produced only pro-IL-18 (24 KDa) in the presence or absence of LPS. However, our study (data not presented) is in agreement with previous studies showing that LPS induced splenocytes and liver cells to produce active IL-18 (18 KDa) [37]. The reason for the difference between our results and those of Strand *et al*., 2005 [26] is not completely clear, but it could be related to the different species used by the two groups (rats by Strand *et al*. 2005 [26], and mice by our group) and/or to detection sensitivity resulting from different methods or reagents used. Different expression of IL-1 in different species has been reported. IL-1 alpha was constitutively expressed in rat [38] and mouse testes [10]; however IL-1 beta was not expressed by rat testis [38] but was expressed in mice testis [10], and in rat testis after stimulation with LPS [39].

Our results suggest an immunological function for IL-18 in the testis, in addition to its physiological functions. IL-18 in the testis could protect against infection and/or inflammation by inducing IFN-γ secretion, NK cytotoxicity and by inducing the expression of several cytokines and Fas ligand [18-21].

In addition, the capacity of LPS to induce IL-18, ICE and IL-18R in the testis and also by LCs under *in vitro* conditions (Huleihel *et al*., data not presented) may indicate a possible direct involvement of IL-18 in the testicular immune response.

Thus, IL-18 could be involved in the regulation of spermatogenesis and may play a role in the local mechanism of the testis as an immune privileged site.

Our hypothesis is that IL-18 could be involved in the regulation of testicular functions during sexual maturation and may control spermatogenesis; this could be under endocrine regulation.

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