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Immune sexual dimorphism: Effect of gonadal steroids on the expression of cytokines, sex steroid receptors, and lymphocyte proliferation

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ABSTRACT

The aims of this study were, first, to explore the differences in the expression of Th1/Th2 cytokines and of steroid receptors in spleen of intact and gonadectomized mice of both sexes; second, to evaluate the effect of estradiol (E2), progesterone (P4) and testosterone (T) on cytokine production and lymphocyte proliferation, and third, to determine the percentage of spleen cell subpopulations in both sexes. Results indicated dimorphic expression of IFN-γ and IL-4, which was affected by gonadectomy. CD4+ T lymphocytes were the most frequent type of cell in the spleen, followed by B lymphocytes (CD19+). Interestingly, there was no dimorphic pattern of cell subtypes, and gonadectomy had no effect. Regarding lymphocyte proliferation, E2 inhibited both cells of male (19.51%) and female (24.62%). P4 diminished lymphocyte proliferation by 22% in cells of female and had no effect on cells of male. It is very interesting to note that the sex steroid receptors mRNA was highly expressed in all splenocytes, and that this expression was dimorphic. However, flow cytometry analysis confirmed that only expression of progesterone receptor was dimorphic. This dimorphic pattern was, however, only seen in lymphocytes. Present evidence indicates that sex steroids are capable of affecting crucial immune system functions dimorphically.

1. Introduction

It has been repeatedly shown that sex, and its associated steroids, significantly influence various aspects of the immune system. A number of reports on immune and neuroendocrine system interactions indicate that hormones are capable of affecting immune functions [1].

The importance of the interaction between the immune and endocrine systems becomes evident in circumstances such as pregnancy, autoimmune diseases and some infectious diseases. In all cases, the available evidence underscores the importance of sex steroids as immunoregulators [2,3].

Although much information remains to be elucidated, these hormones are able to regulate processes implicated in the immune response, including the maturation and selection of thymocytes, cellular transit expression of the major histocompatibility complex class II molecules, lymphocyte proliferation and cytokine production [4]. These functions involve a large repertoire of highly specialized cells that perform different functions with precision and efficacy. Molecules secreted by components of the immune system delicately regulate these cells, but they are also susceptible to regulation by hormones, neurohormones and/or neurotransmitters apparently distant from the immune system. Thus, the system was originally thought to be largely autoregulated, however, it has become increasingly clear that, together with the neuroendocrine system, both systems are directly and bidirectionally interconnected [5]. In this way, physiological systems that integrate complex organisms interact forming networks of mutual control, which favor the correct achievement of their specific functions and of the more general requirements of the complete organism [6]. This interaction plays a relevant role for evolution of vertebrates.

Sex hormones apparently play an important role in the differences in susceptibility associated to sex in certain infectious and autoimmune diseases [7]. Females of different species are known to produce higher levels of circulating immunoglobulins than males, and display a more pronounced humoral immune response against infection. The production of a variety of autoreactive antibodies is also more frequent in females [8]. Estrogens have been found to increase the B cell response both *in vivo* and *in vitro*, while androgens and progesterone diminish antibody production. However, the mechanisms of action of these hormones remain largely unknown. According with these observations, it has been suggested that estrogens potentiate immunity mediated by B cells and suppress some

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mechanisms dependent on T cells [9]. Testosterone seems to suppress both the T and the B cell mediated response [10,11]. In addition to the different immune factors implicated in the regulation of the complex cytokine network, evidence has been found that gender is an important factor in the determination of the secretion pattern of these proteins [12], which suggests that sex steroids may be responsible for these differences. For these hormones to have an effect on immune system cells, the presence of hormone receptors in these cells is necessary. Although steroid hormones also exert effects by non-genomic mechanisms by acting on cell surface receptors and triggering signaling cascades [13,14], it is currently accepted that the main route of biological activity occurs by means of specific nuclear receptors (NR) that function as transcription factors, and coordinate, after binding to their ligand, the expression of target genes [15,16]. The following NR are mediators of these effects: estrogen receptors (ER), ER-α, ER-β, each coded for by an individual gene, whose predominating ligand is E2; progesterone receptor (PR), which has variants A and B generated from the same gene by alternative splicing, whose main ligand being P4, and androgen receptors (AR), coded for by a single gene, its ligands being estosterone (T) and dehydrotestosterone (DHT). One effect of steroids, crucial in the regulator and effector functions of the immune system, and which has not been fully explored yet, is its action on cytokine production and lymphocyte proliferation. According with experimental evidence that supports that host sex is relevant, the object of the present study was to explore the sexual dimorphism of these important aspects of the immune response *in vivo* and*in vitro*.

2. Materials and methods

2.1. Mice and surgical processes

Six week-old BALB/c AnN mice of the two sexes were used in this study. They were fed *ad libitum* with Purina Diet 5015 and water, and kept in light/dark cycle (14 h light:10 h dark). When mice were four weeks old, they were castrated under pentobarbital anesthesia, as reported previously [17] other group received control surgeries (sham) and intact animals were used as controls. Mice were allowed a two-week recovery period before tissue extraction. After this period, mice were sacrificed by cervical dislocation after deep pentobarbital anesthesia and spleen, lymph nodes and gonads were obtained.

Animal care and experimentation practices at the Biomedical Research Institute are frequently evaluated by the University Animal Care and Use Committee and by governmental agencies to ensure compliance with established international regulations and guidelines.

2.2. RNA extraction

Total RNA was isolated from mouse testes, uterus, ovary (positive expression control tissues for sex steroid receptors), spleen and splenocytes by the extraction method using TRIzol reagent (Gibco-BRL, USA). Briefly, each tissue was removed and immediately disrupted in TRIzol reagent (1 mL/0.1 g tissue), and 0.2 mL of chloroform were added per mL of TRIzol. The aqueous phase was recovered after 10-min centrifugation at 15,000 × *g*. RNA was precipitated with isopropyl alcohol, washed with 75% ethanol, and re-dissolved in RNase-free water. RNA concentration was determined by absorbance at 260 nm and its purity was verified after electrophoresis in 1% denaturing agarose gel in the presence of 2.2 M formaldehyde. Total RNA from all extracted tissues was reverse-transcribed, followed by specific PCR amplification of cytokines (IFN-γ, IL-2, IL-4, IL-6) and sex steroid receptors (ER-α, ER-β, PR-A, PR-B, AR) as well as the constitutively expressed gene β-actin.

Table 1
Sequences of the primers used for PCR amplification of total spleen RNA reverse transcribed.

Gene	Sense primer	Antisense primer
IL-2	5′-tgatggacctacaggagctcctgag	5′-gagtcaaatccagaacatgccgcag
IL-4	5′-cgaagaacaccacagagagtgagct	5′-gactcattcatggtgcgacttatcg
IL-6	5′-atgaagttcctctctgcaagag	5′-cactaggtttgccgagtagat
IFN-γ	5′-agcggctgactgaactcagattgtag	5′-gtcacagttttcagctgtataggg
PR-A	5′-cagtggtggatttcatccatg	5′-cttccagagggtaggtgcga
PR-B	5′-ggaggcagaaattccagacc	5′-gacaacaacccttggtagc
ER-α	5′-agactgtccagcagtaacgag	5′-tcgtaacacttgcgcagccg
ER-β	5′-catctgggtatcattacggtg	5′-ggcacttctctgtcttcg
AR	5′-gaatgtcagcctatctttctta	5′-tgcctcatcctcacacactggc
β-Actin	5′-gggtcagaaggattcctatg	5′-ggttctaaacatgatctggg

Primers were designed based on these mouse sequenced genes (Gene databank, NCBI, NIH).

2.3. Retrotranscription-polymerase chain reaction (RT-PCR)

Nucleotide sequences of primers used for amplification are shown in Table 1. Briefly, 4 μg of total RNA from each tissue were incubated at 37 °C for 1 h with 400 units of M-MLV reverse transcriptase (Applied Biosystems, Boston, MA, USA) in 15 μL of reaction volume containing 10 mM of each dNTP and 0.01 μg oligo (dt) primer (Gibco, long Island, NY). Five μL of the cDNA reaction were injected to PCR to amplify specific sequences of the specified genes. The 50 μL PCR reaction included 5 μL of previously synthesized cDNA, 5 μL of 10× PCR-buffer (Biotecnologías Universitarias, UNAM, México City) 30 mM $MgCl_2$, 10 mM of each dNTP, 15 μM of each primer, and 0.5 units of Taq DNA polymerase (Biotecnologías Universitarias, UNAM, Mexico City). Twenty-five μL of the total PCR reaction products of each sample were electrophoresed on 2% agarose gel. PCR products were visualized by staining with ethidium bromide. A single band was detected in all cases, as expected. In order to determine that progesterone receptor (A and B), estrogen receptor (α and β), androgen receptor and each cytokine (IFN-γ, IL-2, IL-4, IL-6) as well as β-actin were in the exponential phase of amplification, and to be assured that changes in these molecules are not artifactual (such as β-actin being in the stationary phase), we performed RNA, cycling and temperature curves for each treatment and tissue studied. The expression of β-actin was used as an internal control at all times and in all tissues studied. In all samples, a single product corresponding to the amplification fragment expected for PR-A (197 nt), PR-B (198 nt), ER-α (251 nt), ER-β (239 nt), AR (365 nt), IFN-γ (247 nt), IL-2 (168 nt), IL-4 (181 nt) and IL-6 (638 nt) and β-actin (238 nt) expression was obtained. After an initial denaturation step at 95 °C for 5 min, temperature cycling was initiated as follows: 95 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min during 25 cycles for PR-A, PR-B and β-actin, 95 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min during 30 cycles for ER-α and ER-β, 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min during 25 cycles for AR, 95 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min during 30 cycles for IFN-γ, 95 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min during 25 cycles for IL-2, and 95 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min during 30 cycles for IL-4 and IL-6. An extra extension at 72 °C was done during 10 min for every gene.

2.4. Densitometric analysis

Hybridization signals were quantified by densitometric scanning of multiple autoradiograms of various exposures, and were represented as the ratio of the signal from the problem gene, relative to the expression of β-actin, a constitutively expressed gene used as internal control (relative expression). The expression of all genes is numerically presented as the ratio of the optical den-

sity (OD) of each studied gene relative to the expression in the same preparation of the β-actin gene, a constitutively expressed gene used as an internal control for differences in the amplification procedure between experiments and in the staining of the different gels. Each fragment was purified and sequenced in order to assure that each band in actual fact belonged to the specified gene.

2.5. DNA sequencing

The DNA sequence was determined by using a Thermo Sequenase cycle sequencing kit (Biorad) and an automatic sequencer (Model LIC-4200, Aloka Co., Japan). The sequence data were analyzed using DNASIS Software (Hitachi Software Engineering, Tokyo, Japan). Homology searching of the nucleotide and protein database was carried out with the Blast program at the National Center for Biotechnology Information (Bethesda, Maryland). Pairwase sequence alignment and protein identities were performed using CLUSTLAW 1.6 software.

2.6. Cell culture and activation

Splenocytes and lymph nodes cells were cultured in serum-free AIM-V media (Gibco-BRL). Splenocytes were cultured, and treated with pharmacological concentrations of E2, P4 and T, in 96-well plates and incubated at 37 °C in humidified 5% CO_2 atmosphere for 76 h. After this time, relative expression of IFN-γ and IL-4 was measured by RT-PCR. Culture grade E2, P4 and T were obtained from Sigma (St. Louis, Misouri). Hormones were dissolved to the desired stock concentration and sterilized by passage through a 0.2 mm Millipore filter. Our concentrations were calculated to resemble those observed *in vivo*, going from lower to higher levels (reaching pharmacological doses). The hormone concentrations used in this study were based upon our previous protocols [17,18] both in mice. Also, studies of serum levels found in mice and other species were chosen to approximate these levels *in vivo*, and to resemble different physiological conditions, such as pregnancy.

For proliferation assays, lymph node cells were isolated, cultured in 96-well plates and incubated at 37 °C in humidified 5% CO_2 atmosphere for 76 h. Cells were activated with anti-CD3 and anti-CD28 antibodies (Pharmingen) and treated with E2, P4 y T. To measure cell proliferation, AlamarBlue dye (Biosource International) was used as reported previously [19]. Briefly, 20 μL/well of AlamarBlue were added to each plate of cultured cells, 24 h prior to the measurement. Absorbance at 600 and 570 nm were measured using a microplate reader.

2.7. Flow cytometry analysis of splenic subpopulations and sex steroid receptor expression

Percentage of subpopulations and steroid receptor expression were detected according to Phosphoflow protocols (BD Biosciences) for intracellular proteins with slight modifications. Briefly, spleenocytes from BALB/c mice were purified and stained with the following antibodies: anti-mCD4-APC, mCD8-PECy5, mCD19-PE, mCD56-PE, mCD11b-bn plus Streptavidin PE (from BD Biosciences). Cells were fixed with 500 μL of Lyse/Fix buffer and then permeabilized with 200 μL of Perm Buffer II. After washing, cells were incubated with Fc blocking reagent (CD16/CD32-FcgammaIII/II Receptor) and incubated with purified anti-ER (estrogen receptor), anti-AR (androgen receptor) or anti-PR (progesterone receptor). Primary antibodies were detected with FITC-coupled secondary antibodies (Zymed). Samples were analyzed by flow cytometry using a FACSCalibur (BD Biosciences). Data were analyzed with the FlowJo software.

2.8. Experimental design and statistical analysis

For the *in vivo* experiments, the experimental design is a three factorial experiment. The independent variables are: (1) sex of which samples were taken (two levels: male or female); (2) Castration (two levels: yes, no) and (3) cell populations (five levels: CD4, CD8, CD19, NK and Mac). The dependent variable is the expression of ER-α, ER-β, PR-A, PR-B, AR, IFN-γ, IL-2, IL-4, IL-6 in the tissue sample, as expressed by the ratio: OD density of the corresponding gel divided by OD density of β-actin in the same tissue sample in the same gel, percentage of cells and mean fluorescence intensity. The β-actin gene is used as control gene for amplification technology. When performed, post hoc individual contrasts of group means by Fisher tests used the sum of Residual and Three Factor Interactions variance to test for significant differences. Differences were considered significant at $P < 0.05$.

In vitro data were analyzed using analysis de variance (ANOVA) with sex (two levels), gonadectomy (two levels) and hormone used in culture (four levels) as independent variables and the dependent variable was proliferation index. If ANOVA revealed significant differences between treatments, the student's *t*-test of differences between groups means was applied to each experiment using the residual variance estimated by ANOVA to test for significance. Data are shown as mean + standard deviation (S.D.), differences were considered significant for $P < 0.05$.

3. Results

3.1. Sexual dimorphism of cytokine expression in spleen

Cytokine expression in the spleen of gonadectomized control mice of both sexes was determined by RT-PCR. For Th1-type cytokines, IFN-γ showed dimorphic expression in both control and gonadectomized animals. In control mice, females expressed more IFN-γ than males, while in gonadectomized mice the pattern of expression was inverted, males showing higher levels than females. IL-2 expression did not differ between control male and female mice; however, in gonadectomized mice, expression was inhibited in males and showed no alterations in females (Fig. 1A). For Th2-type cytokines, IL-4 showed higher levels in males than in females control mice, while gonadectomized mice showed no variations, but expression was inhibited in both sexes compared to control mice. IL-6 expression did not differ between sexes in any of the groups but was decreased in both (Fig. 1B).

3.2. Effect of sex steroids on cytokine expression in spleen cells in vitro

To determine the expression of characteristic Th1/Th2 cytokines *in vitro*, we obtained and cultured splenocytes from male and female mice in serum-free culture medium and evaluated the effect of sex steroids E2, P4 and T on IFN-γ and IL-4 expression. In the first case, we found that, in controls, IFN-γ is more strongly expressed in cells of female than in male and that E2 can inhibit this expression only in cells of female. Treatment with P4 and T had no effect on the basal expression of this cytokine (Fig. 2A). On the other hand, IL-4 expression was also correlated with sex, since females showed higher expression levels than males. In contrast to the effect observed for IFN-γ, in this case, both E2 and P4 can increase the expression level in cells of both sexes. T had no effect on IL-4 expression either (Fig. 2B).

3.3. Effect of sex steroids on lymphocyte proliferation

Lymph node cells were isolated, cultured and activated with anti-CD3 and anti-CD28 antibodies. Then, the effect of the sex

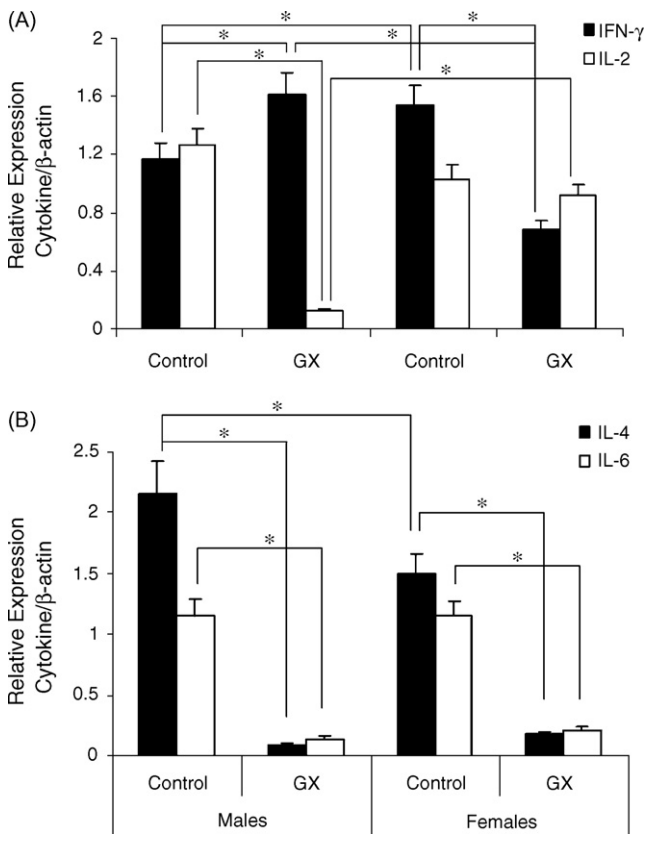

Fig. 1. Sexual dimorphism of cytokine Th1 and Th2 expression in spleen of mice of the two sexes, control and gonadectomized. The results of gene expression are reported as densitometric data of autoradiographic signal. The relative expression was obtained by correcting the expression of each cytokine to that of β-actin. (A) Th1 cytokines, and (B) Th2 cytokines. Data represent the result of 5 mice, and each experiment was done in triplicate. Values are mean + S.D. $^{*}P < 0.05$ compared between sexes. Gonadectomized (GX).

steroids E2, P4 and T in supraphysiological and pharmaceutical concentrations on lymphocyte proliferation was evaluated. We found that lymphocytes of female always showed more proliferation than cells of male. Treatment with E2 at a concentration of 50 nM inhibited proliferation in both cells of, male (19.51%) and female (24.62%) (Fig. 3A); P4 at 62.5 nM concentration diminished proliferation more than 22% in cells of female and had no effect on cells of male (Fig. 3B), and finally, T had no effect on either cells of male or female (not shown).

3.4. Sexual dimorphism of immune cell subpopulations in spleen

In order to show which subpopulations of the different immune cell subtypes are present in the spleen, we decided to determine the percent of each cell type present in this organ, and to analyze if their pattern was dimorphic. In Fig. 4, as expected, the higher percentage of cells present in the spleen are T lymphocytes, mostly CD4+ and B cells (CD19+). Interestingly, only there was a dimorphism in the percentage of macrophages, and they were present in males in numbers almost three times higher than in normal females. Gonadectomy decreased the number of macrophages only in males, compared to intact animals. Our flow cytometry analysis showed that there is no sex-associated pattern of the different cell subtypes, and that gonadectomy did not affect the pattern of different subpopulations, except for the macrophages (Fig. 4).

3.5. mRNA expression of sex steroid receptors in spleen

To determine if the classical mediators of sex steroid effects are expressed in spleen, we searched for the expression of sex steroid

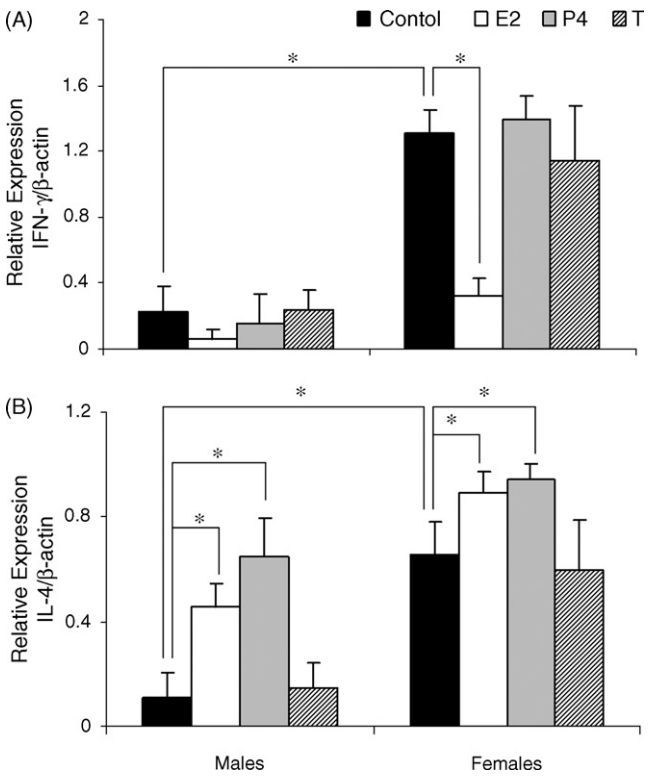

Fig. 2. Effect of sex steroids treatment on the expression of IFN-γ (A) and IL-4 (B) in splenocytes of mice of both sexes. Data are represented as the mean + S.D. of two different experiments ($n = 5$). Each splenocyte culture was done in triplicate. $^{*}P < 0.05$ compared with control cells. Estradiol (E2), progesterone (P4) and testosterone (T).

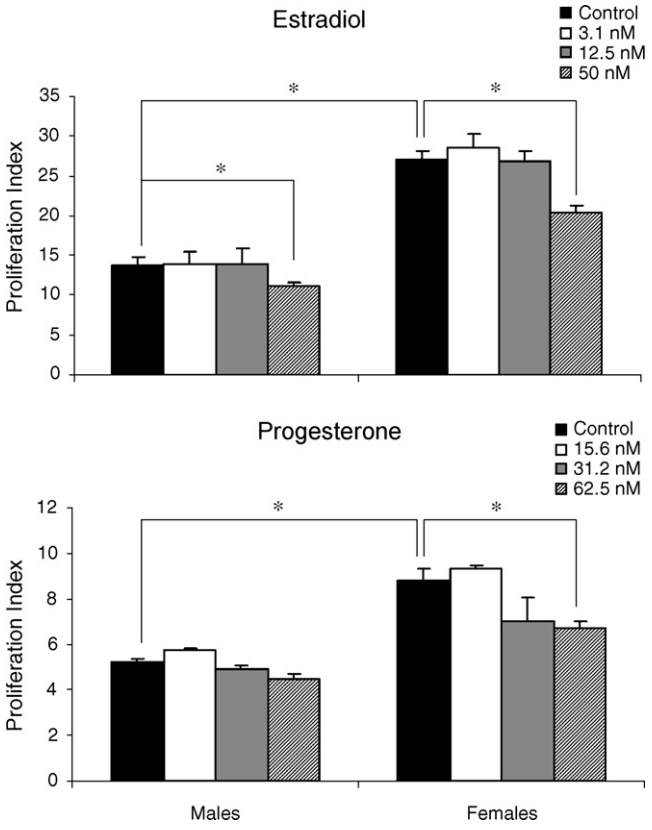

Fig. 3. Effect of estradiol (A), progesterone (B) and testosterone (not shown) on lymphocyte proliferation. Results are shown as proliferation index. Data are represented as the mean + S.D. of two different experiments ($n = 5$). Each cell culture was done in triplicate. $^{*}P < 0.05$ compared with control cells (no steroid added).

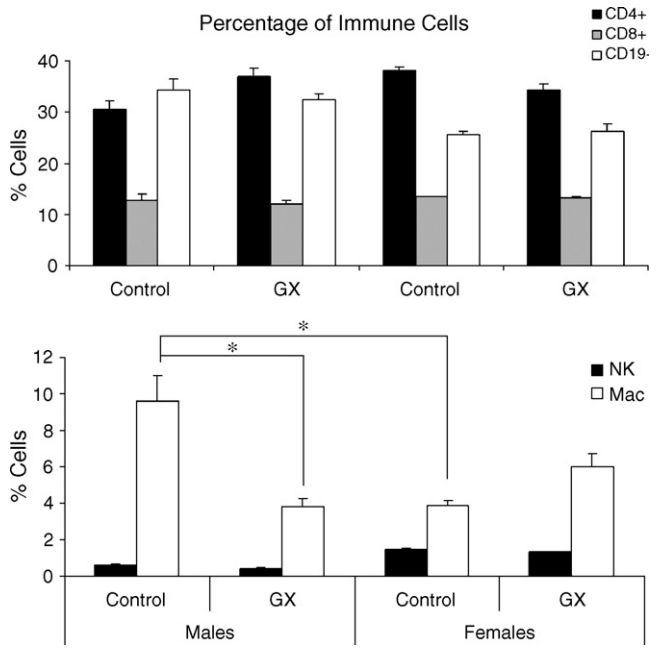

Fig. 4. Percentage of immune cell populations in spleen of mice of both sexes, intact and gonadectomized, detected by flow cytometry. T lymphocytes (CD4+ and CD8 +), B lymphocytes (CD19 +), natural killer cells (NK) and macrophages (Mac) were detected in spleen. Data are represented as the mean + S.D. ($n = 5$). Gonadectomized (GX).

receptors in lymphoid tissue of mice of both sexes. The expression of ER-α and β (Fig. 5A), of PR A and B (Fig. 5B) was determined. Findings were that the spleen expresses the two forms of ER and of PR. We also discovered that all receptors are dimorphically expressed, since, with the exception of PR-A, males express more receptors than females. When the expression of these receptors was measured in gonadectomized animals, we found decreased expression of both ER isoforms just in males; ER-α maintained its dimorphic character. PR-A expression, on the other hand, was inhibited by 50% only in spleen of female, while it remained unchanged in males, and PR-B was inhibited in the two sexes. AR mRNA expression was inhibited by more than 50% only in spleen of male; in spleen of female, gonadectomy had no effects (Fig. 5C).

3.6. Analysis of sex steroid receptors by flow cytometry in splenic subpopulations

The translated functional proteins of ER-α, ER-β, AR and PR were investigated by flow cytometry analysis in different cell subtypes. It is interesting to note that, both ER-β and androgen receptor showed no sex-associated expression (not shown). We found that there are not differences in ER-α expression in different cell types. For PR expression, results showed that its protein levels are clearly dimorphic only in lymphocytes, control males expressing higher (two-fold) PR levels than control females confirming the RT-PCR data. Gonadectomy in males decreased PR expression in T and B lymphocytes by 50% compared to intact animals (Fig. 6).

4. Discussion

The present work explored sexual dimorphism in the expression of Th1/Th2 cytokines in control and gonadectomized mice with interesting results. Firstly, cytokines that are important in the regulation of the immune response, such as IFN-γ and IL-4, show gender-associated differences in control mice. Cytokines secretion is a brief, self-limited event. Cytokines are not usually stored as

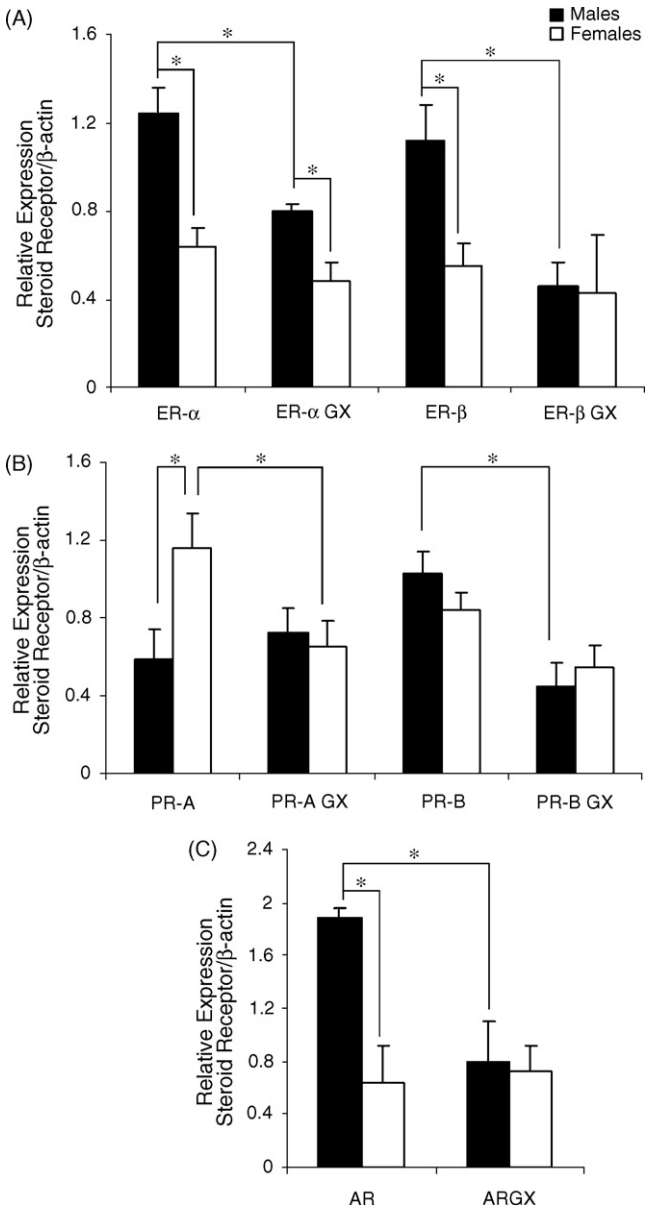

Fig. 5. Sex-steroid receptor expression in total splenocytes of mice of the two sexes, control and gonadectomized. The results of gene expression are reported as densitometric data of autoradiographic signal. The relative expression was obtained by correcting the expression of each steroid receptor to that of β-actin. (A) Estrogen receptors, (B) progesterone receptors and (C) androgen receptor. Data represent the result of five mice, and each experiment was done in triplicate. Values are mean + S.D. $^{*}P < 0.05$ compared between sexes. Gonadectomized (GX), estrogen receptor (ER), progesterone receptor (PR) and androgen receptor (AR).

preformed molecules, and their synthesis is initiated by new gene transcription as a result of cellular activation. Such transcriptional activation is transient, and the messengers RNAs encoding most cytokines are unstable, so cytokine synthesis is also transient. The production of some cytokines may additionally be controlled by RNA processing and by post-transcriptional mechanisms [20], such as proteolityc release of an active product from an active precursor. Once synthesized, cytokines are rapidly secreted, resulting in a burst of release as needed. Thus, it is widely accepted to determine cytokine pattern by studying the mRNA expression only, if no protein data are available. To determine if sex steroids play a role in the expression of these cytokines, we surgically removed the primary source of these hormones, the gonads. We found that, with the exception of IL-2 in females, IFN-γ, IL-4 and IL-6 show signif-

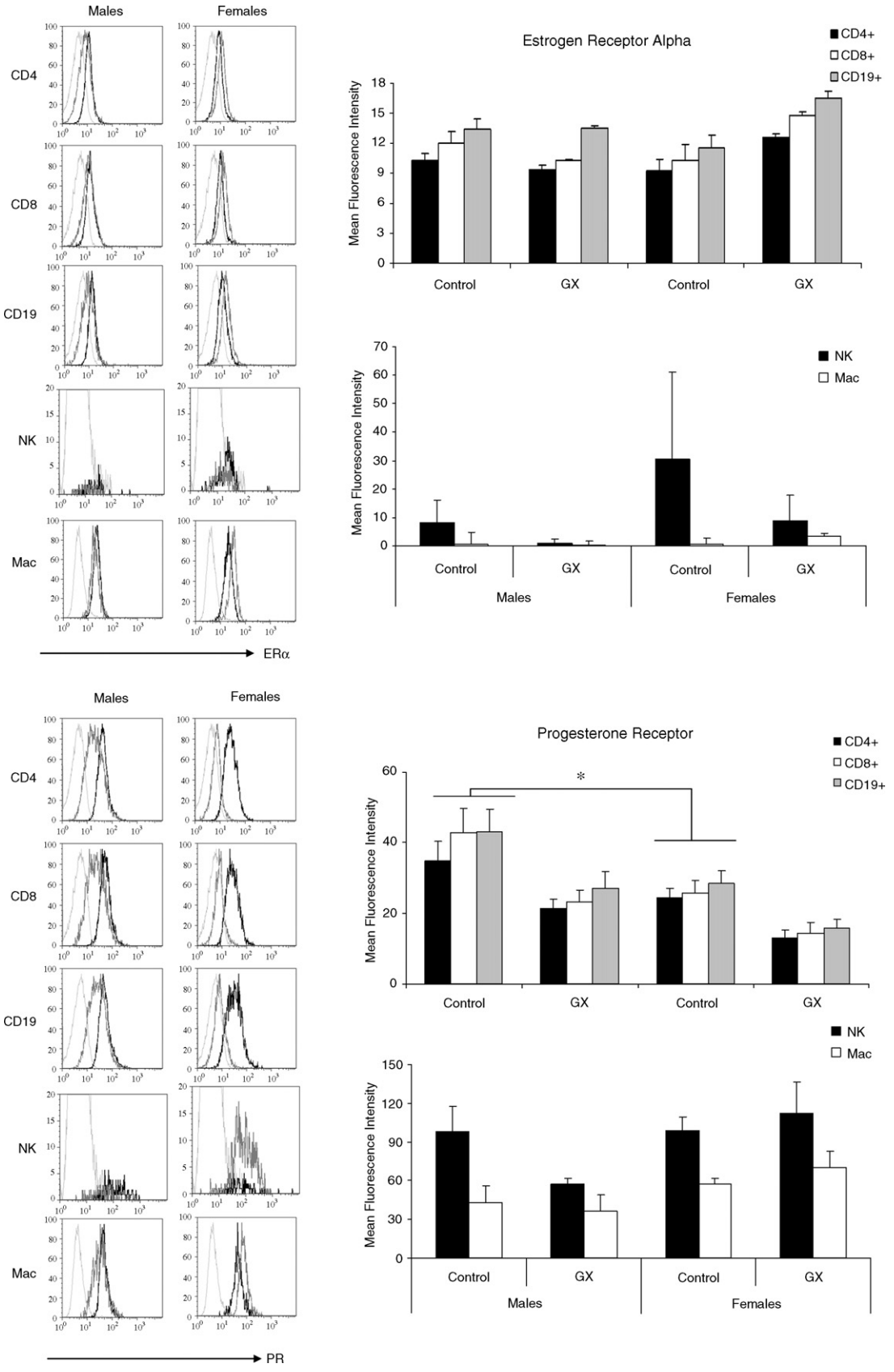

Fig. 6. Sex steroid receptors in spleen cell populations of mice of the two sexes, intact and gonadectomized, detected by flow cytometry. Estrogen receptor alpha and progesterone recptor were detected in T lymphocytes (CD4+ and CD8+), B lymphocytes (CD19+), natural killer cells (NK) and macrophages (Mac). Data are represented as the mean + S.D. ($n = 5$). Gonadectomized (GX).

icant changes, indicating that sex hormones have an effect on the regulation of these cytokines. This is relevant if we consider that these proteins determine the polarization of the immune response towards the effective elimination of pathogens. Our data indicate that the response of male to a particular antigen can be different from the response of female, and thus, the elimination of the antigen will depend, in part, on the sex of the host. It also shows that sex steroids, as modulators of cytokine expression, indirectly affect the combat and elimination of invading organisms.

The effect of sex steroids on cytokine expression was confirmed *in vitro* by isolation and culture of spleen cells to which sex hormones were added. IFN-γ and IL-4 expression was used as markers of the Th1 and Th2 immune response, respectively. Firstly, we confirmed that gender is relevant in the expression of these cytokines, since untreated spleen cells showed sex-associated differences in expression. For both cytokines, females expressed higher levels than males. When cells were treated with sex steroids we found that E2 inhibits the expression of IFN-γ in both, cells of male and female. This agrees with previous work in which E2 is reported to regulate the promoter gene of this cytokine [21]. Contrary to its effect on IFN-γ, E2 induced an increment in IL-4 expression in cells of female and male. The sex-associated difference persisted in cells with and without treatment. In general, estrogens seem to have a dual action on the polarization of the immune response, which depends on steroid concentration. Some studies suggest that estradiol potentiates the production of cytokines Th1 (IFN-γ) and Th2 (IL-10). High estradiol concentrations (>5000 pg mL^{-1}) were found to stimulate IL-10 secretion by T cell clones, while low concentrations stimulate IFN-γ secretion [22,23]. Progesterone also had the effect of increasing IL-4 expression. This agrees with previous work in which the important action of P4 during pregnancy was evaluated [24,25]. Another study indicates that P4 influences polarization of the immune response during the luteal phase of the menstrual cycle. In women with a regular cycle, the immune response during this phase tends towards a Th2-type response, reflected as an increment in IL-4 production [26]. This suggests that the increased P4 and E2 concentrations during the luteal phase play a role in the deviation of the immune response towards a Th2-type response. Testosterone, on the other hand, had no effect on the expression level of these cytokines.

This steroid hormone pattern of action was also observed in the lymphocyte proliferation experiments. Lymph node cells were obtained from mice of the two sexes, then cultured, activated with anti-CD3 and anti-CD28 and treated with E2, P4 and T. Once more, as in the cytokine expression experiments, T had no effect while E2 and P4 inhibited lymphocyte proliferation. Cells of Female proliferated more than cells of male; however, paradoxically, precisely the steroids associated to female physiology were those to inhibit lymphocyte proliferation. A possible explanation to this apparent contradiction is to be found in the age of mice. Six-week old mice were used in all experiments. At this period, mice are not sexually mature yet and, therefore, the hormone changes characteristic of the reproductive phase have not appeared yet. It will thus be interesting to explore the effect of these hormones on lymphocyte proliferation during the reproductive phase. It should also be mentioned that E2 and P4 had different effects on lymphocytes, which depended on the gender of the animal. E2 inhibited cell proliferation in both males and females, while P4 only diminished proliferation in females. If we consider that lymphocyte proliferation can affect the magnitude of the immune response which may subsequently eliminate a pathogen, or not, it follows that progesterone and estradiol have an effect on the eradication of pathogens. Therefore, estradiol is apparently a molecule of great importance not only for the endocrine but also for the immune system.

Considering the effects that steroids exert on the diverse components of the immune system, and that no previous reports are available on the different receptors present in a peripheral organ such as the spleen, we herein aimed to detect the expression of these receptors in the spleen of control and gonadectomized mice. Ample distribution of ER-α and ER-β has been found in thymus, bone marrow and spleen [27–29], however, published reports on the presence of estrogen receptors in lymphoid tissue contrast with the few publications on progesterone receptors. In the present work we found the expression of both types of ER (α and β) and PR (A and B) in the spleen of mice of the two sexes. Gonadectomy, in turn, had significant sex-associated effect on the expression of these receptors. Although the expression level in spleen is much lower than in endocrine tissue, the presence of these ligand-dependent transcription factors is relevant in a secondary lymphatic organ, since it draws attention to the fact that sex steroids may act not only during the maturation and development of immune cells (in thymus) but also during the effector mechanisms of these cells. The question about the population of immune cells that expresses receptors and their regulation was also addressed by flow cytometry analysis.

The possible mechanisms of action of steroids on immune system cells include, as in any classic endocrine tissue, the genomic and non-genomic pathways. According with the genomic action theory, steroids bind to specific receptors present in the cytoplasm, and function as transcription factors. Beside their genomic action, steroids can also act by rapid non-genomic pathways, and the transmission of these effects occurs by specific membrane receptors. Thus, the non-genomic effects on cell function implicate the conventional cascades of second messengers [13]. Although these mechanisms of action have been described in endocrine system organs, evidence has accumulated that they can also operate in the immune system. According to the work by Benten et al. [30], the effects of testosterone on T cells are mediated not only by the intracellular androgen receptor, but also by a membrane androgen receptor on the cell surface. Steroid hormones may act through intracellular and membrane receptors of immune system cells by the non-genomic pathway, whenever the regulation of an immune response against a particular pathogen requires their immediate action, and by the genomic pathway, when the response needs to be delayed. Speculation ensues on the possibility that the non-genomic pathway predominantly regulates the innate immune response, while the genomic pathway does the same with the adaptive immune response.

In addition to the question of which cell population responds to the sex steroids, it would also be interesting to determine the point in time at which the immune system acquires its dimorphic character [31].

The evidence presented above illustrates the importance of immunoendocrine interactions in an immunocompetent host. It strongly suggests an important role for sex steroids in the cytokine network. In practical matters, the complexity of the immunoendocrine interactions suggests that all physiological factors (i.e., sex, age, developmental stage) should be taken into account in the design of vaccines and new drugs. Interventions aimed at the hormonal network appear as a possible new therapeutic approach to control several immune confrontations, such as infections.

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