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Prenatal stress induces up-regulation of glucocorticoid receptors on lymphoid cells modifying the T-cell response after acute stress exposure in the adult life

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HIGHLIGHTS

- Acute stress exposure induced an increase of antibody production in adult mice.
- Prenatal stress (PS) modified the immune response after acute stress in adult life.
- The effect of stress hormones on lymphocyte proliferation was altered in PS mice.
- Corticosterone inhibitory effect was higher on lymphocytes from PS mice.
- An increase of glucocorticoid receptor was found in lymphoid cells of PS mice.

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ABSTRACT

It has been demonstrated that a short-duration stress (acute stress) may result in immunopreparatory or immunoenhancing physiological conditions. The aim of the present study was to investigate whether exposure to prenatal restraint stress (PRS) influences the impact of acute stress on the T-cell response in the adult life. We found that female mice exposed to PRS (PS mice) did not exhibit changes in the T-cell-dependent IgG antibody production with respect to prenatally non-stressed mice (no-PS mice). However, no-PS mice exposed to acute stress showed an increase of antibody production after antigen stimulation. In contrast, PS mice exhibited a decreased response after an acute situation. Spleen catecholamines and plasma corticosterone levels were increased in acute stress in both PS and no-PS mice. Nevertheless, lymphocyte response to hormones was altered in PS mice. Particularly, inhibitory effect of corticosterone was higher on lymphocytes from PS mice. In addition, an increase in protein levels and mRNA expression of glucocorticoid receptor was found in lymphoid cells from PS mice. These results show that prenatal stress alters the immune intrinsic regulatory mechanism that in turn induces an increased vulnerability to any stressful situation able to modify immune homeostasis.

1. Introduction

It has been suggested that exposure to prenatal stress (PS) causes stable long-term changes in central and peripheral stress response systems, and a vulnerability to subsequent adult stress exposure [1,2]. It has also been found that PS induces an enhanced fear-like behavioral profile and dysregulation of brain noradrenergic and hypothalamic–pituitary––adrenal axis (HPA) activity after adult stress exposure [3–6]. Also, maternal social stress during pregnancy affects the reaction pattern of the immune system to social stress in the adult offspring of rats. In response to social confrontation, PS males had a generally lower number of neutrophiles, monocytes, T and NK cells and less lymphocyte proliferation in whole blood cultures than prenatally non-stressed control. Moreover, stress-induced alterations are not quickly reversible in PS males as in control males [7]. However, there are studies in animals [8–11] that suggest that PS has an adaptive effect that helps the offspring to respond appropriately to stressors in the environment. In spite of its pivotal importance for the health of individuals, only few studies have investigated the effects of PS on the immune function. The effects of maternal stress on the innate immunity of the offspring are generally inhibitory (for a review see [12]). It has been described that psychological stress during the third week of gestation decreases macrophage and neutrophil functions, cytotoxicity of blood and spleen natural killer cells, and in vivo resistance to experimentally induced tumors in the offspring [12,13]. PS also modifies the response of T and B lymphocytes to specific antigens. However, the effect depends on the time of application of the stressor during pregnancy, the age of the offspring and the nature of the stressor (see [12]). On the

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other hand, it has been described that acute or short-term stress experiences at the time of immune activation can enhance innate and adaptive immune responses [14,15]. The main mediators of stress effects are norepinephrine and epinephrine, which are released by the sympathetic nervous system, and corticotropin-releasing hormone, adrenocorticotropin and cortisol, which constitute the HPA axis (for a review see [14]). Cells of the immune system, like cells of other organ systems, express receptors for hormones and neurotransmitters. Triggering of these receptors results in the modulation of immune reactivity [16,17].

In this context, the aim of the present study was to investigate whether PS exposure influences the impact of acute stress on the T-cell response in the adult life and the particular interaction between the immune system, the HPA and the ANS under this situation.

2. Methods

2.1. Animals

Sixty-day-old inbred BALB/c mice were purchased from the Veterinary School of the University of Buenos Aires (Argentine). Mice were housed and maintained on an 8:00 AM to 8:00 PM light/dark cycle under controlled temperature (18–22 °C). Animals were handled and sacrificed according to the Institutional Committee for the Use and Care of Laboratory Animal rules (CICUAL, School of Medicine, University of Buenos Aires, Argentine). The experimental protocol was also approved by this committee under resolution 2962/10. The CICUAL adheres to the rules of the "Guide for the Care and Use of Laboratory Animals" (NIH) (2011 revision) and to the EC Directive 86/609/EEC (revision 2010) for animal experiments.

2.2. Prenatal stress

In rodent models PS is generally administered during the final trimester, because this is a critical period in development of the fetal HPA system, and also of potential sensitivity to maternal glucocorticoids [6]. In addition, a large proportion of the immune system development occurs during late gestation (sIg expression on B cell, mitogen responsive thymocytes, pluripotent stem cell seed bone marrow) and the postnatal period (functional NK) in mice [12,18]. For these reasons we choose this developmental time to stress the animals.

Virgin females (10 weeks old) were mated overnight with males on a one-to-one basis. The day on which a copulation plug was found was considered day 1 of gestation. Two groups of 24 pregnant mice each were used through the experiments. The half of pregnant mice were stressed from day 15 of pregnancy until delivery (days 20–21), for 2 h daily (from 10 AM to 12) by placing them in a cylindrical restraint tube (4 cm diameter, 10 cm long) [6,19]. Non-exposed control pregnant females were left undisturbed during all the pregnancy period. Food intake and body weight were not different between control and stressed pregnant mice. Pregnant females gave birth to about four or five pups per litter. The small number of pups found here in both the control and experimental groups is in agreement with that commonly observed when this inbred mouse strain is used [13]. No differences were found between the number of born alive pups, the number of male and female (% of female: 40–60) and the weight at birth between control and stressed pregnant females (weight (g): non-stressed pups: 1.66 ± 0.08; stressed pups: 1.73 ± 0.05). Furthermore, no changes in maternal behavior were observed throughout lactation. Pups from both control and stressed mother were weaned at postnatal day 21 and reared in an environment identical up to the age of 60 days. The female offspring was used for the experiments described below and the male offspring was used for a different set of experiments.

2.3. Stress in the adult life

Both, unstressed (no-PS mice, n = 48) and prenatally stressed female mice (PS mice, n = 48) were randomly assigned to the following groups: acute stress, or undisturbed groups (n = 24 mice for each condition). A schematic diagram of the experimental groups used in this work is shown in Fig. 1. The experimental groups were defined according the pre-natal treatment and the post-natal treatment as follows: mice that received no stress (offspring from unstressed females that received no stress in the adult life, CN), mice from unstressed females that received acute stress in the adult life (AS), mice that received only prenatal stress (PCN), mice that received both prenatal stress and acute stress in the adult life (PAS).

2.3.1. Acute stress

Female offspring of 60 days of age were restrained by placing each animal in a well-ventilated polypropylene tube (2.8-cm diameter–11.5-cm long) for 2 h starting at 10.00 AM. Animals were not physically compressed. Mice were returned to their cages, left undisturbed for 15 min, and then either sacrificed by cervical dislocation (n = 16 of each group) or immunized (n = 8 of each group).

2.4. Immunization

Sheep red blood cells (SRBC, Laboratorio Alfredo C. Gutierrez, Buenos Aires, Argentine) were used as immunogen to evaluate humoral response. For this purpose female offspring of 60 days of age were intraperitoneally (i.p.) immunized (day 0) and boosted 11 days after the first immunization with 0.2 ml of 4% SBRC in saline. The animals were anesthetized in a CO_2 chamber and samples of

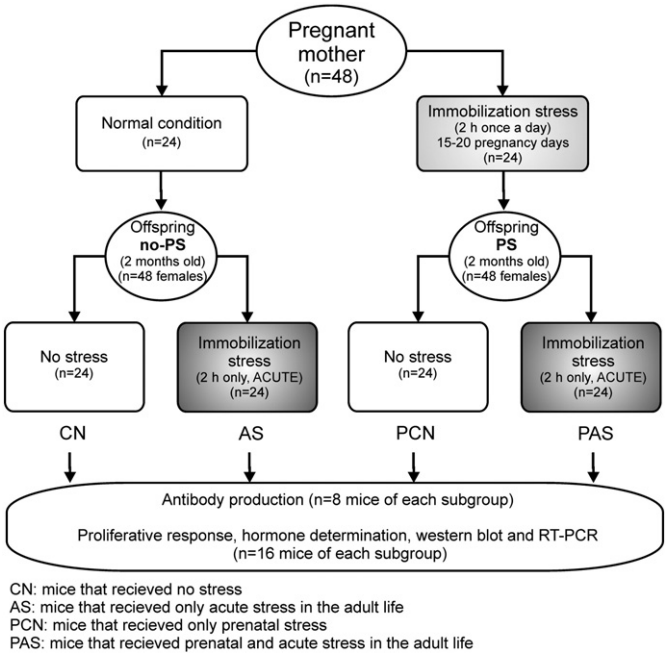

Fig. 1. Scheme diagram of the experimental groups used in the present work. Pregnant mice were distributed in two groups: one was housed in normal conditions (n = 24), and the other was subjected to immobilization stress (n = 24), 2 h daily from day 15 of pregnancy until delivery. The 60-day-old female offspring of each group were divided in four subgroups (n = 24, each one): mice that received no stress (offspring from unstressed females that received no stress in the adult life, CN), mice from unstressed females that received acute in the adult life (AS), mice that received only prenatal stress (PCN), mice that received both prenatal stress and acute in the adult life (PAS). Mice from each subgroup were immunized (n = 8) or sacrificed (n = 16) for hormone determination, evaluation of proliferative response and determination of receptor expression by western blot and RT-PCR.

retro-orbital blood were collected for antibody determination on day 10 (primary response) and on day 18 (secondary response).

2.5. Antibody titres

The serum obtained from retroorbital blood samples was stored at −20 °C until assayed. Quantitative enzyme-linked immunoadsorbent assay (ELISA) was performed to determine SRBC-specific antibodies as previously described [15]. Briefly, 96-well plates (Greiner Bio-One Frickenhausen, Germany) were coated overnight with SRBC membranes (10 μg/ml). Serial-dilutions of sera were added and incubated for 2 h at room temperature, plates were washed, and samples were incubated with a goat IgG anti-mouse IgM or anti-mouse IgG phosphatase alkaline conjugated (Sigma-Aldrich, St. Louis, MO, USA) and p-nitrophenylphosphatase (Sigma-Aldrich, St. Louis, MO, USA) as substrate to develop coloration that was read at 405 nm. Reactions were considered positive when optical density (OD) values were above the mean value plus 2 SD of normal sera (sera from non-immunized vehicle-injected mice that gave non-statistical differences among them). Antibody titer for each serum was determined as the last serum dilution with positive reaction.

2.6. Cell suspensions and culture conditions

Lymph node (inguinal, axial and mesenteric) cell suspensions from mice under different experimental conditions were obtained as previously described [15]. Briefly, lymph nodes were removed and disrupted through a 1-mm metal mesh, and the cell suspension was filtered through a 10-μm nylon mesh. After three washes in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Grand Island, New York, USA), cells were resuspended in RPMI 1640 supplemented with 10% of batched-tested non-stimulatory fetal calf serum (Internegocios S.A., Buenos Aires, Argentine), 2 mM glutamine (Gibco, Grand Island, New York, USA), 100 U/ml of penicillin (Gibco, Grand Island, New York, USA), 100 μg/ml of streptomycin (Gibco, Grand Island, New York, USA), and 50 μM ß-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA).

2.7. Mitogen assay

Proliferation was determined by culturing 3×10^5 cells per well in 96-well plates in a final volume of 0.2 ml in triplicate aliquots of supplemented medium. The T-cell mitogen Concanavalin A (Con A; Sigma-Aldrich, St. Louis, MO, USA) was added to the microcultures in different concentrations (from 0.5 to 2 μg/ml). Then, cells were cultured at 37 °C in a 5% CO2 atmosphere for different periods (from 2 to 4 days). Mitogenic activity was measured by adding 1 μCi [^{3}H]-thymidine (20 Ci/mmol, Perkin Elmer Inc., Waltham, MA, USA) per well for the last 18 h of culture. Thymidine uptake was measured by scintillation counting after retention over GF/C glass-fiber filters (Whatman, Brentford, UK) of the acid insoluble macromolecular fraction. Mitogen-stimulated cells displayed the expected proliferation kinetics, with a peak of proliferation on the third day of culture. To analyze the influence of catecholamine or corticosterone on the proliferative response, co-incubation was carried out with epinephrine (Sigma-Aldrich, St. Louis, MO, USA) or corticosterone (Sigma-Aldrich, St. Louis, MO, USA), each at concentrations ranging from 1×10^{-10} M to 1×10^{-4} M. For this purpose hormone was added with Con A at the start of the culture. Freshly prepared stock solution of corticosterone (4×10^{-2} M) in dimethyl sulfoxide and epinephrine in culture medium (10^{-2} M) was used. When cells were treated with corticosterone the final concentration of DMSO in the culture medium was less than 0.25%.

2.8. Catecholamine assay

As previously described, to evaluate SNS activation we determined catecholamine levels in spleen, which receive an important sympathetic innervations [20]. Catecholamine concentrations were determined in samples from mice under different experimental conditions by the fluorometric assay described by Laverty and Taylor [21]. Briefly, spleens were homogenized in 12.5% sodium sulfite, and 10% EDTA in 0.4 N perchloric acid. After 24 h at 4 °C, homogenates were centrifuged at 15,000 *g* for 15 min. Supernatants were brought to pH 8.2 and seeded in a pre-washed alumina column (Sigma-Aldrich, St. Louis, MO, USA). Eluates were oxidized with iodine in an alkaline medium. The fluorescence was recorded at 480 nm in a spectrofluorometer (Jazco FP 770) using an excitation source of 380 nm. Sensitivity of catecholamine assay was 0.025 and 0.08 μg/total homogenate, for norepinephrine and epinephrine respectively. Recovery was 86 ± 4 and 77 ± 4% for norepinephrine and epinephrine respectively. The range of the assay was 10–100 μg/μl, in which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity. The correlation coefficients were greater than 0.9995, indicating adequate linearity.

2.9. Corticosterone determination

To avoid fluctuations in plasma corticosterone levels due to the circadian rhythm, mice were bled at 12:15 PM on the day of the sacrifice. Blood from the animals under the different experimental conditions was collected on ice in 0.25 M EDTA and separated in a refrigerated centrifuge. Plasma was stored at −80 °C until the assay was performed. Corticosterone levels were determined using a standard radioimmunoassay [22]. [1,2,6,7-^{3}H(N)-corticosterone] (20 Ci/mmol) was from Perkin Elmer Inc. (Waltham, MA, USA). The antibody (Sigma-Aldrich, St. Louis, MO, USA) cross-reactivity with other relevant steroids was 4.5% (cortisol), 20% (deoxycorticosterone) and 7.9% (testosterone). Sensitivity of the corticosterone radioimmunoassay was 300 pg/ml and intra- and inter-assay coefficients of variation (CVs) were 5.4 ± 1.7 and 8.9 ± 0.7%, respectively.

2.10. Western blot

Lymphoid node were homogenized in buffer (20 mM HEPES, 1 mM DTT, 1 μM leupeptin, 1 μM PSMF, 0.2 μM L-valine). Homogenates were centrifuged at 10,000 rpm for 10 min. Supernatants were supplemented with a 3× sodium dodecyl sulfate (SDS) sample buffer (2% SDS, 10% (v/v) glycerol, 62.5 mmol/L Tris–HCl, pH 6.8, 0.2% bromophenol blue, 10 mmol/L 2-mercaptoethanol). Proteins were separated by SDS-PAGE on 12% polyacrylamide gels and transferred to nitrocellulose membranes. Non-specific binding sites were blocked with blocking buffer (5% non-fat dried milk, containing 0.1% Tween-20 in 100 mmol/L Tris–HCl, pH 7.5 and 0.9% NaCl) for 1 h. Membranes were subsequently incubated with rabbit anti-mouse glucocorticoid receptor or rabbit anti-mouse β_2-adrenergic receptor (Santa Cruz Biotechnology, Santa Cruz, Texas, USA) for 24 h. Anti-β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, Texas, USA) was used as control of equal loading and transfer efficiency. Then membranes were incubated for 1 h with HRP-conjugated goat anti-rabbit immunoglobulin (Ig) G as secondary antibodies (1:2000; Santa Cruz Biotechnology, Santa Cruz, Texas, USA). Membranes were analyzed by enhanced chemiluminescence (Amersham Biosciences, UK). Precision Plus Protein Kaleidoscope Standards (Bio-Rad, CA 94547, USA) was used as molecular weight markers. The optical density (OD) of the bands was determined by quantitative densitometry with a computerized image processing system (ImageQuant TL 7.0, GE Healthcare, Uppsala, Sweden). The densitometric analysis was performed using Gel-Pro Analyzer System (Version 3.1.00.00, Media Cybernetics, Inc. Rockville, MD 20850, USA). The optical density (OD) of β-actin bands was used as a normalizing factor.

2.11. RT-PCR

Total RNA was extracted from whole lymphoid node by homogenization in Trizol Reagent (Invitrogen, Life technologies, California, USA) and total RNA was isolated following manufacturer's instructions. The total RNA was used as a template to generate first-strand cDNA synthesis using the M-MLV Reverse Transcriptase (Invitrogen, Life technologies, California, USA), random primers (Invitrogen, Life technologies, California, USA) and dNTPs (Invitrogen, Life technologies, California, USA). The cDNA (2.5 μg) was used for PCR amplification with 1× of Go-Taq® DNA polymerase (Promega Corporation, Madison, USA), and dNTPs (Invitrogen, Life technologies, California, USA) at 25 mM each, in a DNA thermal cycler (T personal thermocycler, Biometra, Goettingen, Germany). The sequences of mouse specific primers, annealing temperature and number of PCR cycles are described in Table 1. Sequence amplification was done using a thermocycler (T personal thermocycler, Biometra, Goettingen, Germany). PCR products were visualized on 1% agarose gels by ethidium bromide staining (Promega Corporation, Madison, USA). Relative gene expression was quantified by densitometry with the Gel-Pro Analyzer System (Version 3.1.00.00, Media Cybernetics, Inc. Rockville, MD 20850, USA). Values were referred to β-actin as a housekeeping gene for data normalization, because no significant differences between groups using cyclophilin or glucose-6-phosphate-dehydrogenase (G6PDH) mRNA expression levels were found (data not shown). Reactions were performed in triplicate and the results were averaged.

2.12. Statistical analysis

Data were analyzed using two-way ANOVA to examine the significance of the main effects [pre-natal treatment (normal or PS) and post-natal treatment (no stress or acute stress in the adult life)] and interactions [pre-natal treatment × post-natal treatment]. When interaction was significant, simple effect analysis (F) was made. The antibody production data were not normally distributed, so the non-parametric statistic Kruskal–Wallis (H) test was performed. A two-way ANOVA for repeated measures for in vitro study of the dose–response to corticosterone/epinephrine was used. Differences between means were considered significant if $p < 0.05$. Statistical and data analyses were performed using GraphPad Prism version 5.00 for Windows.

3. Results

3.1. IgM and IgG antibody production after T-dependent antigen immunization in no-PS and PS adult mice subjected to acute stress

To investigate whether PS exposure was associated with changes in antibody production, we examined the humoral response to SRBC, a thymus-dependent antigen, in no-PS and PS mice that were either stressed or not in the adult life. Fig. 2A shows that the anti-SRBC IgM primary response (day 10) was not significantly different between groups [H (3) = 2.625, NS]. In contrast, anti-SRBC IgG titres (day 18) were significantly different between groups [H (3) = 24.83, $p < 0.0001$]. Post-hoc analysis showed that antibody production was not affected by PS exposure (CN *vs* PCN, NS) (Fig. 2B). However, antibody production between non-exposed and exposed mice to stress in the adult life in both PS and no-PS mice was significantly different. In no-PS mice the antibody production was higher in mice that received acute stress (AS, Fig. 2B, $p < 0.05$) than in non-exposed mice (CN). However, PS mice showed a significant decrease in antibody response after acute stress (PAS *vs* PCN, $p < 0.05$ and PAS *vs* AS, $p < 0.001$, Fig. 2B).

3.2. T-cell reactivity in normal and PS adult mice subjected to acute stress

To analyze the effect of PS exposure on mitogen-induced proliferative response of T cells, dose–response curves with the T mitogen were performed with lymphocytes from normal and PS mice subjected or not to acute stress in the adult life (see Fig. 3). Two-way ANOVA showed that the proliferative response between groups was significantly different depending on pre-natal treatment (no-PS or PS) and post-natal treatment (no stress or acute stress in the adult life) [interaction: pre-natal treatment × post-natal treatment; F (1,20) = 9.77, $p < 0.01$]. Simple effects analysis indicated that the proliferative response was not altered in PCN mice with respect to non-exposed mice (CN) [F (1,20) = 0.44, NS], but showed differences between post-natal treatment in no-PS [CN vs AS, F (1,20) = 11.16, $p < 0.01$] but not for PS [PCN vs PAS, F (1,20) = 1.16, NS] mice.

3.3. Influence of catecholamines and corticosterone on normal and PS adult mice exposed to acute and chronic stress

To investigate the participation of catecholamines and corticosterone in the disruption of the T-cell response, we evaluated the levels of these hormones after stress exposure (Fig. 4). The two-way ANOVA showed that the levels of corticosterone (Fig. 4A) between groups were significantly different depending on the post-natal treatment [interaction: pre-natal treatment × post-natal treatment: F (1, 36) = 2.68, NS; post-natal treatment: F (1, 36) = 138.37, $p < 0.0001$]. Post-hoc analysis revealed that acute stress induced a significant increase in serum corticosterone level in both normal (CN vs. AS, t (18) = 9.475, $p < 0.001$) and PS (PCN vs. PAS, t (18) = 7.160, $p < 0.001$, AS vs. PAS, t (18) = 1.49, NS) mice. With respect to splenic catecholamine content (Fig. 4B), the two-way ANOVA showed a significant difference between groups depending on the post-natal treatment [interaction: pre-natal treatment × post-natal treatment F (1, 36) = 0.01, NS; post-natal treatment: F (1, 36) = 22.30, $p < 0.0001$]. Post-hoc analysis revealed that acute stress induced a significant increase in splenic catecholamine content in both no-PS (CN vs. AS, t (18) = 3.281, $p < 0.01$) and PS (PCN vs. PAS, t (18) = 3.398, $p < 0.01$; AS vs. PAS, t (18) = 0.19, NS) mice. In addition, we analyzed the effect of corticosterone and catecholamines on lymphocyte reactivity. Fig. 5 shows the effect of corticosterone and epinephrine on mitogen-induced T-cell proliferation in no-PS and PS mice. The addition of corticosterone resulted in a dose-dependent modulation of the proliferative response [two-way repeated measures ANOVA, F (3,24) = 70.51, $p < 0.0001$] dependent on the steroid concentration for both no-PS and PS mice (Fig. 5A). The corticosterone effect was different depending on the pre-natal treatment [CN vs PCN: F (1,24) = 18.79, $p < 0.0001$]. Thus, low concentrations of corticosterone induced a stimulatory effect and high concentrations exert an inhibitory effect on proliferative response of lymphocytes from no-PS mice. However, the stimulatory effect was not observed on proliferative response of lymphocytes from PS mice (Fig. 5A). In addition high concentrations of corticosterone exert a greater inhibitory effect on the proliferative response of lymphocyte from PS in comparison

Table 1
Specific primers, annealing temperature and number of PCR cycles.

Gene	Forward sequence (5′ → 3′)	Reverse sequence (5′ → 3′)	Size of PCR product (bp)	Annealing temperature (°C)	Number of PCR cycles
GR	CCAAGGGTCTGGAGAGGAC	CTGGACGGAGGAGAACTCAC	123	57	36
β_2-adrenergic receptor	TCGAGCGACTAC AAA CCG TC	CCAGAACTCGCACCAGAAGT	146	57	36
β-actin	CAACTTGATGTATGAAGGCTTTGGT	ACTTTTATTGGTCTCAAGTCAGTGTACAG	97	61	30

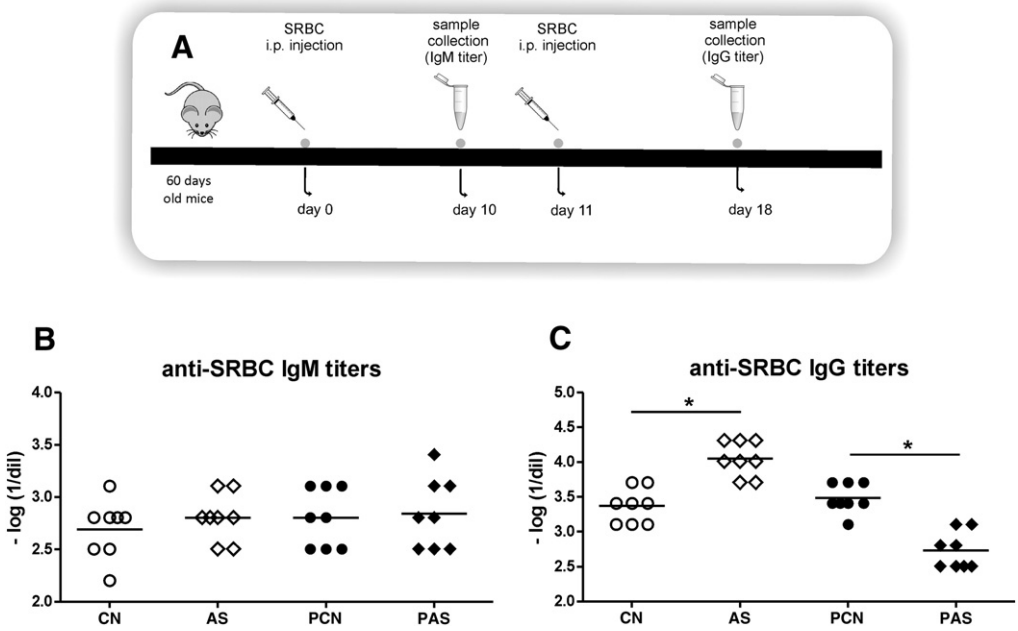

Fig. 2. Antibody titers following SRBC immunization in no-PS and PS mice. Panel A: schematic representation of the experiment timeline. Prenatally unstressed mice (no-PS), unstressed (n = 8, ○) or stress-exposed in the adult life (acute n = 8, ◇) and PS mice non-exposed (n = 8, ●) or stress-exposed in the adult life (acute n = 8, ◆) were immunized with SRBC, and 4% saline, as indicated in the Methods section. Sera were collected on day 10 and day18 and assayed for the presence of IgM (panel B) or IgG (panel C) by ELISA respectively. $^{*}p < 0.05$ with respect to the corresponding non-postnatally stressed mice.

to those no-PS mice (Fig. 5A). Moreover, epinephrine (Fig. 5B) significantly modulated mitogen-induced T-cell proliferation of normal lymphocytes depending on the concentration [two-way repeated measures ANOVA, $F(3,24) = 63.34$, $p < 0.0001$], but not differences were observed according the pre-natal treatment [CN vs PCN: $F(1,24) = 0.14$, NS]. Similar results were obtained for norepinephrine (data not shown).

3.4. Expression of glucocorticoid and ß$_2$-adrenergic receptor on lymphocytes from normal and PS adult mice

We analyze if changes in corticosterone response induced by prenatal exposure were due to regulation of corticosteroid receptor expression. As can be seen in Fig. 6 both, mRNA expression and protein levels in lymphoid cells from PS mice were significantly increased with respect to no-PS mice ($t(8) = 3.860$, $p < 0.01$ and $t(8) = 3.565$, $p < 0.01$, respectively). In accordance with epinephrine effect on lymphocyte proliferation, both mRNA expression and protein levels of β_2-adrenergic receptor were not altered by PS exposure ($t(8) = 0.360$, NS and $t(8) = 0.317$, NS, respectively).

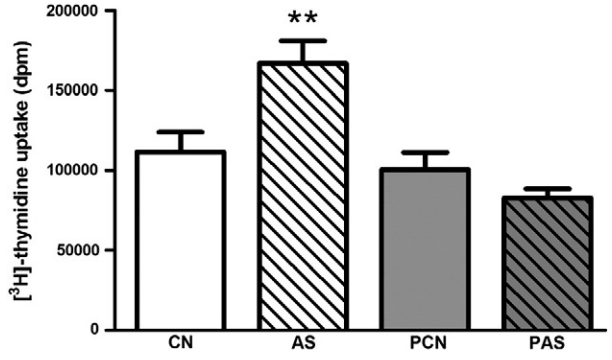

Fig. 3. Cellular proliferation assay. Mitogen-induced proliferation of T-lymphocytes no-PS (white bars) and PS (gray bars) mice was determined by [^{3}H]-thymidine uptake (dpm). Results show the mean ± SEM of the maximal ^{3}H-thymidine uptake (3rd day of culture and 1μg/ml of Con A) in lymphocyte culture from mice unstressed in the adult life (CN, n = 6 and PCN, n = 6) or mice exposed to acute stress (AS, n = 6 and PAS, n = 6) in the adult life. $^{**}p < 0.01$ with respect to the corresponding non-postnatally stressed mice. [^{3}H]-thymidine uptake (dpm) for non-stimulated cells was: CN: 2238 ± 429, AS: 2551 ± 380, PCN: 2515 ± 542 and PAS: 3051 ± 546.

4. Discussion

This study demonstrates that exposure to prenatal stress (PS) modifies the magnitude and the pattern of stress-induced immune system changes in the adult female offspring.

According to our previous results, the humoral response to a T-cell dependent antigen is increased after acute stress exposure [15]. Here, we found that animals exposed to PS showed no changes in the IgG antibody production in response to a T-cell dependent antigen immunization, but a decreased humoral response after acute stress exposure. As IgM production was not affected, it is possible to postulate that the T-cell dependent isotype switching is mainly affected by adult stress exposure in PS mice. In addition, we found similar results for the in vitro T-cell dependent proliferative response.

The studies investigating the consequences of maternal stress over the immune system of the offspring have shown variable results. The PS effects depend on the animal species, the nature of the stressor, the duration of stress, the intensity and persistence of the stressor, as well as the immune compartments investigated and the age of animals. In particular, in rodent models, several studies reported immunosuppressive [13,23] and immunoenhancing [24] effects of various PS procedures in rats. In addition, some authors observed no effects of PS on the humoral immune response of neonate and juvenile rats [25] or both immunosuppression and increased production of pro-inflammatory cytokines in juvenile rats [26]. Kinetic studies revealed that the effect of PS strongly depends on the age of the offspring [27]. However, there is no explanation yet why the time when these effects appear after birth is variable between studies and why this expression is often delayed in time [12]. In this context, it has been postulated that PS induces early developmental changes of immunity that would no longer persist after weaning; nonetheless, these transient early

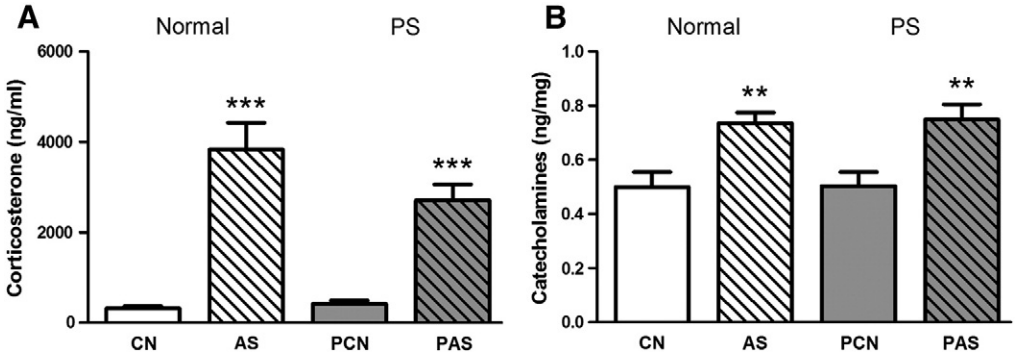

Fig. 4. Serum corticosterone levels and catecholamine concentrations in spleen samples from no-PS (white bars) and PS (gray bars) in non-exposed (CN and PCN) and acute stress (AS and PAS) exposed mice. Results for corticosterone are showed in panel A and represent the mean ± SEM of ten mice of each group. Results for catecholamine are showed in panel B and represent the mean ± SEM of 10 mice of each group. $^{**}p < 0.01$, $^{***}p < 0.001$ with respect to their corresponding non-postnatally stressed mice (CN and PCN).

modifications could later alter the immune-reactivity of the organism towards infections [27]. So, it is possible to assume that PS has long-term implications on the immune function inducing an increased vulnerability to any stressful situation able to modify immune homeostasis. Our results indicate that PS female mice have an impaired immune response after acute stress with respect to no PS mice. In accordance, Götz et al. [7] showed that in response to social confrontation, the immune alterations are not quickly reversible in PS male rats in contrast to control males.

Regarding the role of catecholamines and corticosterone in stress effects, their levels are increased under acute situations as expected. In rodents and non-human primate species, it has been found that PS causes alterations in the hypothalamic-pituitary-adrenocortical (HPA) axis and in the brain neurotransmitter systems in the offspring for review, see [1,28]. Although PS research on HPA function shows diverse results, most studies have found some alteration in the HPA axis in glucocorticoid response. It has been suggested that the HPA alterations induced by PS may vary according to gender, as well as to the nature and intensity of the stressor for a review, see [29]. This altered adaptation is characterized in male PRS rats by reduced hippocampal plasticity, with decreased glucocorticoid receptor density, while female PS rats seem to be better shielded from the effects of PS on hippocampal plasticity [29].

It has been proposed that immune alteration induced by PS may be a consequence of an altered receptor density on immune or/and endothelial cells caused by differential exposure to stress hormones such as corticosterone during intrauterine development. However, PS-associated changes of hormone receptors on immune cells have not been previously investigated. Our results show for the first time that the lymphocyte response to corticosterone on mitogen-induced proliferation in PS mice was altered, thus corticosterone induced a greater inhibitory effect on lymphocytes from PS mice in comparison to those from no-PS mice. Several studies have demonstrated that corticosterone and epinephrine are important mediators of an acute stress-induced immunoenhancement for a review, see [14]. Low-dose corticosterone or epinephrine administration significantly enhance skin delayed-type hypersensitivity (DTH), whereas high-dose corticosterone, chronic corticosterone, or low-dose dexamethasone administration significantly suppresses skin DTH. According to our results, it is possible that a dysregulation in lymphocyte responses to corticosterone and catecholamines results in an inadequate response to antigen challenge after acute stress exposure. Dhabhar and his group have done an extensive research on the effects of stress on immune response (for review see [14,30]). This authors note that critical factors (such us, the duration and magnitude of stressor, or stress hormone exposure relative to the time of activation and ensuing time course of the immune response) are likely to influence the direction (enhancing versus suppressive) of the effects of stress or stress hormones and the nature of the immune response (immunoprotective, immunopathological, or immunoregulatory/suppressive). Our results suggest that PS exposure can act on the immune system of the progeny by directly altering the immune intrinsic regulatory mechanism that in turn induces an impairment ability to properly respond under acute stress situations. Thus, PS constitutes an important factor that could influence the effect of stress hormones on the immune response. In addition, the present

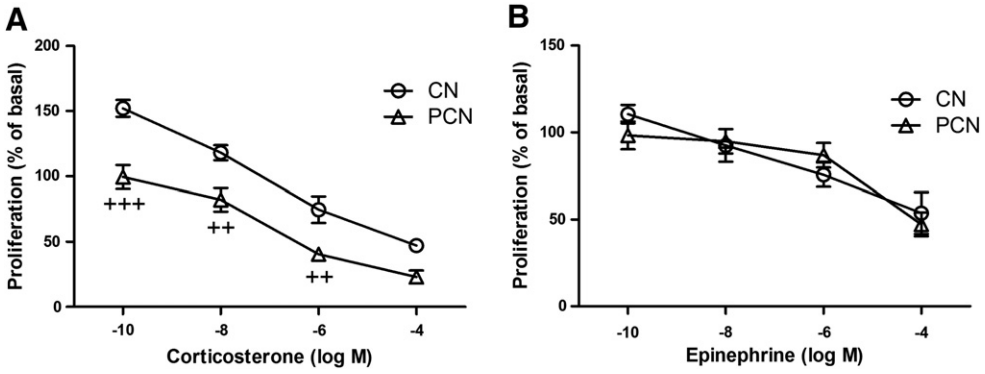

Fig. 5. Effect of epinephrine and corticosterone on mitogen-induced T-cell proliferative response. Con-A stimulated T-lymphocytes from no-PS (CN) and PS (PCN) mice were co-incubated with corticosterone (Panel a) and epinephrine (Panel b), each at concentrations ranging from 1×10^{-10} M to 1×10^{-4} M. Results are expressed as the percentage of the thymidine uptake in the absence of corticosterone or epinephrine (basal proliferation). Results represent the mean ± SEM of six mice of each group. $^{++}p < 0.01$, $^{+++}p < 0.001$ respect to the corresponding non-prenatally stressed mice.

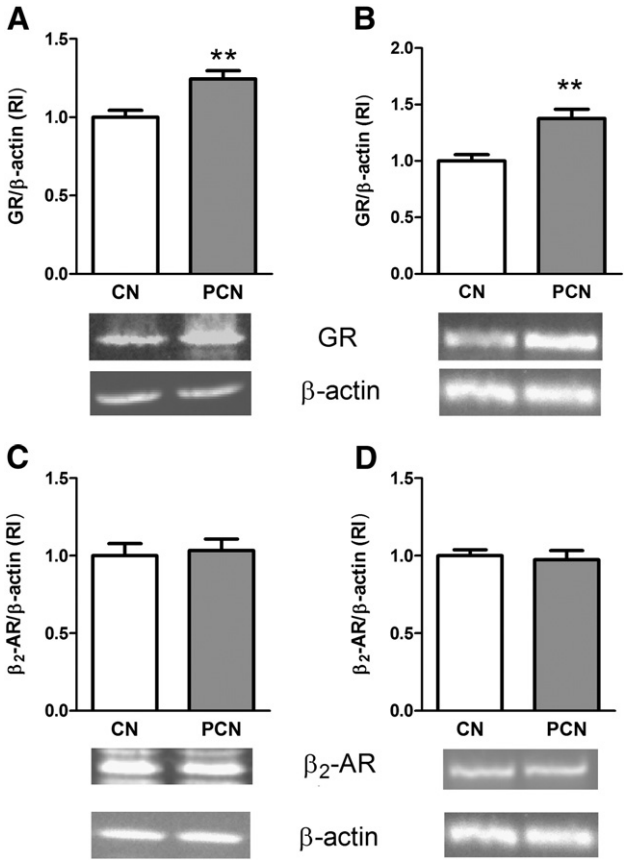

Fig. 6. Glucocorticoid receptor (GR, top panel) and β_2-adrenergic receptor (AR, down panel) protein and mRNA levels. Western blot (A and C) and PCR (B and D) analyses were performed in lymphoid node homogenates from control (CN) and prenatal stressed (PCN) mice. The bands shown are representative of five independent experiments. The graphics indicate the mean ± SEM of the relative intensity (RI) for each receptor with respect to β-actin. The control group was normalized to a mean value of 1. $^{**}p < 0.01$ with respect to control.

results highlight a novel and important functional significance of stress hormone receptors on lymphocytes in PS.

5. Limitations

The present study was lead to explore if PS modified the effect of acute stress on the immune response. One important limitation is that this study was carried out only in female. As male mice are known to fight and get hurt when are kept in one cage, which constitutes a strong stressor, this first attempt to study the effect of PS on the stress-induced immune alteration in the adult life was performed with females. However, it is important to extend this study using male and female mice to determine if gender differences exist in the effect of PS on immune system. Another important question is that glucocorticoid and β-adrenergic receptor levels were determined in lymph node. It is known that lymph node has a higher percentage of T-lymphocytes. Nevertheless, further studies are needed to correlate the up-regulation of glucocorticoid receptors on T-cells induced by PS and alterations of T-cell response after acute stress exposure.

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