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Neonatal testosterone imprinting affects thymus development and leads to phenotypic rejuvenation and masculinization of the peripheral blood T-cell compartment in adult female rats

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ABSTRACT

Exposure of female rodents to testosterone in the critical neonatal period produces defeminization/masculinization of the hypothalamo–pituitary–gonadal (HPG) axis, i.e. neonatal androgenization and postpones axis maturation. To address the hypothesis that HPG axis signaling is involved in the programming of thymic maturation/involution and sexual differentiation we studied the impact of neonatal androgenization on thymic cellularity, development of effector and regulatory T cells, and phenotypic characteristics of peripheral blood T lymphocytes in adult rats. A single injection of testosterone on postnatal day 2 postponed thymic maturation/involution as revealed by organ hypercellularity, increased cellularity of the most mature (CD4+CD8− and CD4−CD8+) TCR$\alpha\beta^{high}$ thymocyte and both recent thymic emigrant (RTE) subsets and caused phenotypic defeminization/masculinization of thymic (decreased CD4+CD8−TCR$\alpha\beta^{high}$/CD4−CD8+TCR$\alpha\beta^{high}$ cell ratio) and peripheral blood T-cell compartments (decreased CD4+RTE/CD8+RTE and CD4+/CD8+ cell ratio). In addition, neonatal androgenization increased the relative and absolute numbers of both CD4+CD25+Foxp3+ and natural killer (NK) regulatory T cells in peripheral blood. These findings, in conjunction with thymocyte overexpression of Thy-1 that is assumed to reduce negative selection affecting self-reactive cell generation, suggest a new relationship between self-reactive and regulatory T cells. In conclusion, our study provides additional evidence for a role of HPG signals (i.e. sex steroids and gonadotropins) in programming the kinetics of thymic maturation/involution and in establishing immunological sexual dimorphism.
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1. Introduction

Given that there is a strict synchrony between the organization and development of hypothalamic centers involved in the regulation of gonadal function and the generation of immunocompetent cells (Pierpaoli et al., 1977; Allen et al., 1984), close interdependency between the development of hypothalamo–pituitary–gonadal (HPG) and thymo-lymphatic axes has been suggested (Pierpaoli et al., 1977; Allen et al., 1984). Estrogens, acting via intracellular (Staples et al., 1999; Yellayi et al., 2000; Erlandsson et al., 2001) and membrane G protein-coupled receptor 30 (GPR30) receptors (ERs), (Wang et al., 2008) promote thymus growth early in life and induce organ involution beginning around puberty (Staples et al., 1999; Yellayi et al., 2000; Erlandsson et al., 2001) leading to gradual decay in immune responsiveness (Berzins et al., 1998).

There is sexual dimorphism in many thymic functional indices as well as in the ratios of mature thymocyte subsets (Leposavić et al., 1996; Shames, 2002; Pilipović et al., 2008). This dichotomy is believed to trace the route for sexual dimorphism in the immune response (Luster et al., 1984; Ansar Ahmed et al., 1999; Shames, 2002). Compared with males, females have an elevated CD4+/CD8+ T-cell ratio and exhibit enhanced immunoreactivity (Grossman, 1985; Kovacs and Olsen, 1998) providing better protection against infections, but also enhanced autoreactivity contributing to the induction of autoimmunity (Kovacs and Olsen, 1998; Ansar Ahmed et al., 1999; Shames, 2002). There is a consensus that sex steroids, acting primarily at the thymic level, are partly responsible for this phenomenon (Kovacs and Olsen, 1998; Shames, 2002). It has been suggested that intracellular ER-β is required not only for full estrogen-mediated thymic involution in female mice (Erlandsson et al., 2001) but possibly for development of thymic sexual dimorphism (Wang et al., 2008). Due to the fact that both the aromatase system converting androgen to estrogen and ERs are detectable in the rat thymus

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during the critical period and that the aromatase inhibitor during this period causes sexually dimorphic effects on ER density in both the thymus and hypothalamus, sexual differentiation of these tissues has been speculated to proceed during the same critical period and via similar estrogen-mediated mechanisms (de Fougerolles Nunn et al., 1999).

It has long been thought that the brain of rodents, and perhaps all mammals, is inherently feminine or bipotential (Barraclough and Gorski, 1961; Arnold and Gorski, 1984; Breedlove et al., 1999). There is also general agreement that in the absence of testicular androgens during the critical period (late prenatal and the first 10 postnatal days) brain regions controlling reproductive function develop passively in a primarily female manner, while the presence of these hormones leads to their defeminization/masculinization (Gorski, 1971; Arai et al., 1986). Testosterone administration to female rats during the critical period postpones sexual maturation as revealed by delayed vaginal opening and causes the development of polyfollicular non-ovulatory ovaries devoid of the corpora lutea with hypoprogesteronemia and hyperestrogenemia followed by impaired estrogen responsiveness (Gerall and Kenney, 1970; McEwen et al., 1977; Arai et al., 1986).

Given that developmental disturbances in the HPG axis affect thymic development and that sexual differentiation of the HPG axis and thymus depends on the presence of testosterone during the critical period, then in neonatally androgenized rats postponed thymic maturation/involution and defeminization/masculinization (leading to phenotypic remodelling of the peripheral T-cell compartment) may be expected. To test this hypothesis in neonatally androgenized adult rats, we examined both thymic cellularity and thymocyte maturation by estimating cellularity and the relationships among the main thymocyte subsets (identifiable by characteristic expression of surface CD4, CD8 and TCR antigens), as well as surface density of Thy-1 which regulates thymocyte selection thresholds (Hueber et al., 1997).

In the thymus CD4−CD8− double negative (DN) TCR-precursors undergo successive stages of development confronting fateful decisions: the pre-TCR checkpoint (β selection), positive and negative selection (von Boehmer et al., 1989). The first developmental step involves TCRβ locus rearrangement and surface expression of the pre-TCR. Downstream signaling from the pre-TCR (β selection) induces survival and surface expression of CD4, CD8 and TCRαβ (Zamoyska and Lovatt, 2004). Thereafter, these CD4+CD8+ double positive (DP) TCRαβ^{low} thymocytes are scrutinized for their ability to recognize peptides in the context of self-MHC expressed on thymic non-lymphoid cells. Only DP cells transducing moderate TCRαβ signal strength (positive selection) continue to differentiate into mature CD4+CD8− or CD4−CD8+ single positive (SP) TCRαβ^{high} cells that emigrate from the thymus forming a pool of recent thymic emigrants (RTEs) in the periphery. All other cells terminate differentiation either by being eliminated by negative selection or dying through neglect. Thymic selection provides generation of a peripheral T-cell repertoire that can respond appropriately to pathogens while displaying tolerance to peripheral self-antigens. However, under physiological conditions a minority of self-reactive thymocytes attains the SP stage and escapes into the periphery. These cells in the periphery are under continuous control of CD4+CD25+Foxp3+ and NKT T regulatory (Treg) cells, which are also produced by the thymus (Seddon and Mason, 2000; Hori et al., 2003; Berzins et al., 2004). Therefore, to fully assess the impact of neonatal androgenization on the thymus in peripheral blood from neonatally androgenized rats not only the proportion and numbers of RTEs and T effector (Teff) subsets were estimated but also those corresponding to Treg cells.

2. Materials and methods

2.1. Animals

Neonatal rats born to inbred Albino Oxford (AO) strain females bred in the animal house facility at the Immunology Research Centre in Belgrade were used. The animals were randomly assigned to either neonatal testosterone administration (NA group) or vehicle administration (Control group). Three individual experiments were performed each involving at least 5 animals per experimental group. Pre-weaning rats were housed with their mothers under standard laboratory conditions. At postnatal (p.n.) day 21, when weaning was completed, littermates were removed from their mothers.

2.2. Experimental protocol

At p.n. day 2 (day 0 = day of birth) rats in the NA group were administered with testosterone enanthate (TE, 1000 μg, s.c.). The dose was chosen based on previous studies (Suzuki et al., 1998; Watanobe and Habu, 2003) and our preliminary experiments to avoid multiple injections, and to provide a pronounced delay in vaginal opening, constant vaginal estrus, hyperestrogenemia and hypoprogesteronemia. Control rats received an equivalent volume (50 μl) of vehicle (purified olive oil). After weaning the success of the treatment was confirmed by regular vaginal opening examination . All control rats showed vaginal opening between p.n. days 30 and 35. As previously reported, all TE-administered rats on p.n. day 80 required surgical vaginal opening using small scissors (Watanobe and Habu, 2003). All animals were euthanized by exposure to increasing doses of CO_2 followed by cardiac puncture exsanguination (between 09:00 and 10:00 h). Estrous cyclicity was assessed by analyzing vaginal smears (lavages) on a daily basis between 08:30 and 10:30 h from day 80 onwards using a light microscope. Due to practical constraints, the oil-injected (control) animals were not all at the same cycle stage at autopsy. In agreement with previous reports (Suzuki et al., 1998; Watanobe and Habu, 2003), every TE-injected rat showed constant vaginal estrus. At autopsy, in the ovaries of oil-treated rats follicles at various stages of development and large corpora lutea were evident. The ovaries of the rats exposed to androgens were polyfollicular and lacked corpora lutea.

The minimal number of animals required to perform the study and all the procedures involving animals and their care were approved by our Institutional Animal Care and Use Committee which follows principles laid down in the European Community's Council Directive dated the 24th November 1986 (86/609/EEC).

2.3. Serum estradiol and progesterone levels

Blood samples were taken by cardiac puncture. The serum was separated and stored at −20 °C until the analyses. Serum estradiol was determined using the IMMULITE solid-phase competitive chemiluminescent enzyme immunoassay (EIA) on an IMMULITE 1000 analyzer (Euro/DPC, UK) according to the guidelines provided by the kit producer. Serum progesterone was measured by radioimmunoassay (RIA) using a commercial kit (INEP Dijagnostika, Zemun, Serbia).

2.4. Chemicals, antibodies and immunoconjugates

Testosterone enanthate was obtained from Galenika A.D. (Zemun, Serbia). Sodium azide and concanavalin A (ConA) were purchased from Sigma-Aldrich (Taufkirchen, Germany).

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