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AGE-ASSOCIATED ALTERATIONS IN SYMPATHETIC NORADRENERGIC INNERVATION OF PRIMARY AND SECONDARY LYMPHOID ORGANS IN FEMALE FISCHER 344 RATS

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Abstract

Normal aging processes, as well as, psychological stress affect the immune system; each can act alone, or interact with each other, to cause dysregulation of immune function substantially altering physical and mental health. The sympathetic nervous system (SNS), a major mediator of stress effects on immune function, is significantly affected by normal aging process, and stress can affect aging of the SNS. Previously, we have shown age-associated changes in sympathetic noradrenergic (NA) innervation of lymphoid organs in male rodents that affect immune regulation. The purpose of this study was to investigate sympathetic innervation of lymphoid organs and associated alterations in immune responses in young and aging female Fischer 344 (F344) rats. Histofluorescence and immunocytochemistry for NA innervation, and neurochemistry for norepinephrine (NE) levels were performed in the thymus, spleen, and mesenteric lymph nodes (MLN) isolated from 3-month-old young (normal estrous cycle), 8- to 9-month-old (onset of irregular estrous cycling), and 24–25 month, and 30–31 month female F344 rats (acyclic) at diestrus based on vaginal smears. Age-related alterations in natural killer (NK) cell activity, interleukin-2 (IL-2) and interferon- γ (IFN- γ) production, T and B lymphocyte proliferation were examined in splenocytes. Sympathetic NA innervation and NE levels increased with aging in the thymus, declined in spleen and MLN, and was accompanied by significant reductions in NK cell activity, IL-2 and IFN- γ production, and T and B cell proliferation in old female rats. In 8–9 mo rats, NE levels in the hilar region of the spleen and IFN- γ production were unaltered, while NE levels in the end region of the spleen and IL-2 production were reduced. Collectively, these results suggest that aging is characterized by significant alterations in sympathetic NA innervation in the thymus, spleen, and MLN associated with immunosuppression, and that there is a marked shift in NA activity and immune reactivity occurring during middle-aged female rats.

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Keywords

Tyrosine hydroxylase; Norepinephrine; Immunity; Cytokines; Natural Killer cell activity; Lymphocyte proliferation; Thymus; Spleen; Mesenteric lymph nodes

1. Introduction

Aging in females is associated with changes in the levels of gonadal hormones and immunosuppression resulting in menopause and subsequent development of osteoporosis, autoimmune diseases, cancers, and infectious diseases (Dawson-Hughes, 2008; Tanriverdi et al., 2003; Anisimov et al., 2003; Curns et al., 2005). Immune reactivity is regulated by neuroendocrine outflow through hormones and sympathetic innervation of the lymphoid organs. Several earlier studies from our laboratory have demonstrated the presence of sympathetic noradrenergic (NA) nerves in primary and secondary lymphoid organs of male mice and rats, and that these nerves release norepinephrine (NE) to influence immune responses (Felten et al., 1987; Bellinger et al., 2001a; Madden, 2001). NA sympathetic nerve fibers in primary and secondary lymphoid organs undergo age-associated alterations in their density, distribution in the parenchyma of lymphoid organs, NE concentrations, NE uptake and release, and β -adrenergic receptor-induced signaling in rodents (Bellinger et al., 1988; Bellinger et al., 1992; Felten et al., 1987; Bellinger et al., 2001b; Bellinger et al., 2008; Perez et al., 2009). In general, thymic involution is associated with increased NA nerve fiber density and NE concentration, while there is a decline in NA neuronal density and NE concentrations in spleen and lymph nodes accompanied with immunosuppression in male rats and mice (Bellinger et al., 2001b).

Thus far, age-associated alterations in sympathetic NA innervation of lymphoid organs and their link to immunosenescence have not been investigated in females, especially during the various stages of estrous cycles during young and middle age or during acyclicity in old female rats. Female rats attain puberty around 35–40 days of age, exhibiting a regular 4-day cycle with 4 distinct stages based on secretion of sex hormones: proestrus, estrus, metestrus (diestrus I), and diestrus (diestrus II). At 8–10 months of age, the estrous cycle is irregular with an extra day of estrus or diestrus II. This stage is followed by a constant estrus stage (10- to 19-month), marked by high circulating estrogen (E). Constant estrus is followed by persistent diestrus, with increased circulating progesterone (P), and then by anestrus (19- to 30-month; Lu, 1983; Wise, 1982). These progressive alterations in estrous cycle patterns with increasing age are associated with the development and growth of mammary tumors and autoimmune diseases (Meites, 1980; McCombe et al., 2009). Altered secretion of pituitary hormones, including luteinizing hormone (LH), follicle-stimulating hormone, and prolactin (PRL), and the ovarian hormones, E and P regulates the 4 distinct stages of the estrous cycle in young rats. In early middle-aged rats, the onset of the LH surge in the afternoon of proestrus is delayed, its amplitude is reduced (Lu, 1983; Wise, 1982), and the pulsatility of LH release is altered (Wise, 1982) causing irregular estrous cycles. Fluctuations in circulating gonadal hormones during the various stages of estrous cycle influence T and B cell proliferation, and localization of IgA-producing plasma cells (Krzych et al., 1978). Age-associated reduction in natural killer (NK) cell activity, T cell proliferation, and mitogen- or antigen-induced interleukin-2 (IL-2) production were observed in female rats (Davila and Kelley, 1988).

In the present study, we investigated the pattern of sympathetic NA innervation and NE concentrations in thymus, spleen, and mesenteric lymph nodes (MLN) collected during diestrus II stage in young (cyclic), early middle-aged (irregular cycling), and persistent diestrus stage in old female rats. Simultaneously, immune responses including NK cell

activity, concanavalin A (Con A)-induced proliferation of T lymphocytes, Con A-stimulated IL-2 and interferon- γ (IFN- γ) production in splenocytes, and lipopolysaccharide (LPS)-induced B cell proliferation were also measured to determine the changes in immune reactivity across the various age groups. We report here that in female rats, there is an age-related decline in sympathetic NA innervation in spleen and MLN along with decreased immune responses beginning with the early middle age.

2. Materials and Methods

2.1. Animals

Young (3-month; $n=8$), early middle-aged (8–9 months; $n=6$), and old (24–25 months; $n=10$; and 30–31 months; $n=12$) female Fischer 344 (F344 rats) were purchased from Charles River Laboratories, obtained through Roche Bioscience, Palo Alto, CA. Rats were housed two per cage in a temperature-, humidity-, and light-controlled (12:12-hr light/dark cycle) animal room, and were provided with food and water ad libitum. Animals were observed for changes in physical condition and/or presence of age-related illness. All animal experiments were conducted in accordance with the principles and procedures outlined in the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, and were approved by the institutional Animal Care and Use Committee.

After a 10-day acclimatization period, estrous cycles were monitored daily by vaginal cytology for 10 days. Young and early middle-aged rats that showed two consecutive estrous cycles were sacrificed on the day of diestrus II, while the old rats were sacrificed during the persistent diestrus (continuous diestrous smears) stage. At the time of sacrifice, all visceral organs were autopsied for evidence of gross pathologies and tissues were dissected for study. Rats with any visible lesions, tumors and evident pathology were removed from this study and their tissues were excluded from analyses. The animals were decapitated, and spleens were removed aseptically and cut into four equal blocks for histofluorescence for catecholamines, neurochemistry for NE, and immunological assays, as described previously (ThyagaRajan et al., 2000). The center blocks of tissue with the entry point for splenic artery and sympathetic nerves into the spleen were designated as hilar region, while the two distal blocks farthest from the hilus were referred to as end regions. The center two hilar blocks and one distal block of spleen tissue were frozen on dry ice, and stored at -80°C until further analysis for fluorescence histochemistry and high-performance liquid chromatography with electrochemical detection (HPLC-EC). The remaining distal block of spleen was used for immunological assays, including Con A-stimulated IFN- γ and IL-2 production, Con A-induced proliferation of T lymphocytes, LPS-induced proliferation of B lymphocytes, and NK cell activity. Thymus and MLN were removed for histofluorescence for catecholamines and neurochemical analysis of NE by HPLC-EC. A separate set of animals were used for immunocytochemistry (ICC) analysis for tyrosine hydroxylase (TH; the rate-limiting enzyme in the synthesis of NE),

2.2. Fluorescence histochemistry

The glyoxylic acid condensation method (SPG method) of histofluorescence for catecholamines (de la Torre, 1980) was used to visualize NA sympathetic nerves in lymphoid organs from female rats across age. A piece of thymus, the MLN and the hilar region of the spleen were sectioned at $16\text{ }\mu\text{m}$ on a cryostat maintained at -20°C . Sections were thaw-mounted onto glass slides, dipped in sucrose-potassium phosphate-glyoxylic acid solution, and dried for 20 min under a direct stream of cool air. Sections were covered with mineral oil and placed in an oven for 3 min at 95°C . Then the oil was drained from the slides, the slides were coverslipped with a drop of mineral oil, and stored at -5°C until

further analysis. All the tissue sections were photographed using a Nikon fluorescence microscope equipped with epi-illumination accessories.

2.3. ICC for tyrosine hydroxylase (TH)

A separate set of female rats from each age group were anesthetized with Chloropent (0.4 ml/100 g body weight) and perfused transcardially with 100 ml of phosphate buffer (pH 7.2) containing 0.9% sodium nitrite and heparin followed by 300 ml of 4% paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.2). Spleens were dissected, cut into 4 equal blocks, postfixed in the perfusion fixative for 24 h at 4°C, and transferred into phosphate buffer with 30% sucrose for an additional 24 h at 4°C. Subsequently, spleen blocks were frozen on dry ice and stored at -80°C until sectioning. For sectioning, the hilar block of spleen was mounted onto the freezing chuck of an AO sliding microtome using PBS, 30 µm-thick sections were cut, transferred into 24-well plates containing cryoprotectant solution, and stored at -20°C. ICC was carried out using a polyclonal rabbit anti-TH antibody (1:750; Chemicon, Temecula, CA) with nickel sulfate intensification of 3,3'-diaminobenzidine (DAB) chromagen reaction product. All steps were carried out in phosphate buffer (pH 7.2) at 25°C using gentle agitation, unless otherwise indicated. Sections were rinsed and incubated for 30 min in 10% normal goat serum (NGS). The primary antibody was diluted 1:750 in phosphate buffer containing 0.4% Triton X-100 and 0.25% bovine serum albumin. Incubation in the primary antibody was carried out at 4°C overnight with gentle agitation. Control sections were incubated in phosphate buffer in the absence of the primary antibody.

The next day, sections were rinsed 6 × 10 min, incubated for 30 min in 10% NGS, and then incubated in secondary goat anti-rabbit antibody (Vector Elite kit) diluted in buffer (1:6000) for 90 min. Sections were rinsed 4 × 10 min and incubated in 2.5% methanol with 8% hydrogen peroxide for 30 min to remove endogenous peroxidase activity. Following 6 × 10 min rinses, sections were incubated in an avidin biotin peroxidase complex (ABC; Vector Elite kit; 1:8000 dilution) for 90 min. Sections were rinsed 4 × 10 min, followed by 2 × 10 min in 0.05 M acetate imidazole buffer, pH 7.2, and then developed in acetate imidazole buffer containing 0.25 g/100 ml nickel (II) sulfate, 0.04 g/100 ml DAB, and 0.005% hydrogen peroxide for 15–20 min. Sections were rinsed 2 × 10 min in acetate imidazole buffer and 4 × 10 min, mounted on gelatin-coated slides, dried, dehydrated through a graded series of ethanol, cleared in xylene, and cover-slipped in Permount. Subsequently, all sections were visualized and photographed for using a light microscope.

2.4. HPLC-EC

Prior to analysis by HPLC-EC, NE in the thymus, spleen, and MLN was extracted with alumina. Tissues were homogenized in 0.1 M of HClO₄ containing 0.25 µM of 3,4-dihydroxybenzylamine (DHBA) as the internal standard and were centrifuged at 1000 g for 5 min. The supernatants were used for aluminum oxide extraction, and the pellets were saved for protein assay (Bio-Rad assay kit; Bio-Rad, Hercules, CA). At the time of HPLC-EC analysis, samples were loaded onto a Waters 717plus autosampler (Waters, Milford, MA), and NE concentration was measured using HPLC-EC, as described previously (ThyagaRajan et al., 2000). NE concentration in the thymus, spleen, and MLN was expressed as pmoles/mg protein and pmoles/mg wet weight of the tissue. NE content in the whole spleen was calculated using NE concentration/total splenic wet weight in mg in the combined hilar and end region of the spleen.

2.5. NK cell activity

Lymphocytes from the spleen were prepared as described previously (ThyagaRajan et al., 2000). Cells were resuspended to the desired concentration in RPMI 1640 medium supplemented with 5% fetal calf serum (Sigma Chemical Co., St. Louis, MO), 1 mM sodium

pyruvate, 2 mM L-glutamine, 0.01 mM nonessential amino acids, 5×10^{-5} M 2-mercaptoethanol, 100 U/ml penicillin, 100 mg/ml streptomycin, 24 mM sodium bicarbonate, and 10 mM HEPES for *in vitro* culture.

NK cell activity was assessed using the NK-sensitive lymphoma, YAC-1, passaged *in vitro*. YAC-1 cells in log phase growth were incubated with 100 μ Ci of $\text{Na}_2^{51}\text{CrO}_4$ (DuPont NEN, Boston, MA) at 37°C for 90 min. The cells were washed three times and adjusted to 10^5 cells/ml. Spleen cells were mixed with 10^4 ^{51}Cr -labeled YAC-1 cells at varying effector to target (E:T) ratios in round-bottom, 96-well tissue culture plates (Falcon, Becton Dickinson, Oxnard, CA) in triplicate in a total volume of 200 μ l. Spontaneous release was determined by incubating 10^4 ^{51}Cr -labeled YAC-1 cells with complete RPMI alone. Maximum release was determined by adding 1% Triton X-100 to 10^4 ^{51}Cr -labeled YAC-1 cells. The plates were then centrifuged at 200 g for 5 min and incubated for 4 h at 37°C in a 5% CO_2 humidified incubator. The plates were centrifuged at 500 g for 5 min at 4°C, 100 μ l of supernatant was removed from each well, and radioactivity was counted in a gamma counter. Cytotoxic activity was expressed as percent lysis, determined by the equation (experimental cpm-spontaneous cpm)/(maximum cpm-spontaneous cpm) $\times$ 100.

2.6. Assay for IL-2 and IFN- γ production

Lymphocytes (2×10^5 cells/well) were incubated with either medium alone or 1.25 μ g/ml of Con A in 24-well tissue culture plates (Falcon, Becton Dickinson). After 24 h of culture, 1 ml of supernatant was removed from each well and stored at -20°C until assayed for cytokine content. To measure IL-2, supernatants were tested for the ability to support the growth of the IL-2-dependent cell line, CTLL-2. Supernatants from individual wells were serially diluted and incubated with 4×10^3 CTLL-2 cells in a total volume of 100 μ l for 40 h. Cell growth was then determined using a colorimetric assay as previously described (ThyagaRajan et al., 2000). Ten μ l of 5.0 mg/ml 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) in Hank's balanced salt solution (HBSS) was added to each well. After incubating for 4 h at 37°C, 100 μ l of 0.04 N HCl in isopropanol was added to dissolve the colored precipitate. Absorbance was measured with a microplate reader (Bio-Tek Instruments, Winooski, VT) using a test wavelength of 570 nm and a reference wavelength of 630 nm. Background values (CTLL-2 cells incubated with medium only) were subtracted automatically from each value.

IFN- γ levels in supernatants were determined by ELISA. ELISA plates (Corning, Corning, NY) were coated overnight at 4°C with purified anti-rat IFN- γ polyclonal antibody (1 μ g/ml; Biosource International, Camarillo, CA) in 0.1 M Na_2HPO_4 buffer (pH 9.0). In between steps, plates were washed with PBS containing 0.05% Tween-20 (PBS/Tween). Plates were then blocked for 2 h with PBS-10% fetal equine serum (FES) at room temperature. Recombinant rat IFN- γ (Biosource) or samples serially diluted in culture media were added to plates in triplicate and incubated overnight at 4°C. Biotin-conjugated anti-rat IFN- γ (0.5 μ g/ml) diluted in PBS-10% FES was added to each well, and the plates were incubated at room temperature for 1 h. Avidin-peroxidase (Sigma), diluted 1:400 in PBS-10% FES, was added to the plates, and incubated for 30 min at room temperature. In the final step, substrate ABTS (2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid; Sigma) containing 0.03% hydrogen peroxide was added to the plates and incubated for 30 min at room temperature. Absorbance at 405 nm was measured with a microplate reader (Bio-Tek Instruments) after 30 min.

2.7. Con A- and LPS-induced proliferation

Spleen cells, 2×10^5 cells/well, were cultured in triplicate with either medium alone or varying concentrations of Con A (Calbiochem-Behring Corp., La Jolla, CA), in 96-well,

flat-bottomed tissue culture plates (Falcon), and maintained for 3 days at 37°C in a humidified 5% CO₂ incubator. [³H]-Thymidine (0.5 µCi/10 µl; 5 Ci/mmol; DuPont NEN, Boston, MA) was added for the final 18 h of culture. Cells were harvested onto glass fiber filter paper (Whatman Inc., Clifton, NJ) with a cell harvester (Skatron, Norway). The dried filters were placed in scintillation fluid (Biosafe II, RPI, Mount Prospect, IL), and radioactivity determined with a liquid scintillation counter (LKB, Wallac, Finland). LPS-induced proliferation of B lymphocytes was measured in a similar manner as Con A-induced proliferation, except that the spleen cells were incubated with medium or a single concentration of LPS (5 µg/ml).

2.8. Statistical analysis

The data were analyzed by one-way analysis of variance (ANOVA). Con A-induced proliferation and E:T ratios for NK cell activity was analyzed using ANOVA with Con A concentration as repeated measures. Parameters that attained significance with ANOVA ($P < 0.05$) were further analyzed by Fisher's least significant difference test.

3. Results

3.1. Age-associated increase in NA innervation in the thymus

In the young rats, sympathetic NA nerve fibers enter the thymus as nerve bundles through the capsule or arteries and travel in plexuses surrounding blood vessels in the capsule, septa, and subcapsular zone. The distribution of NA nerve fibers was observed throughout the cortex with Strands of nerve fibers branch out from these plexuses into the cortical and paracortical parenchyma where thymocytes reside. Medullary innervation was characterized by the presence of nerve fibers around the medullary venous sinuses, thick plexuses around the thymic arteries and arterioles, and sparse innervation in the parenchyma.

Age-related thymic atrophy was associated with deposition of fatty tissues among the thymic parenchyma (data not shown). There was slight increase in the NA innervation in the cortical and paracortical regions of the thymus from the 8- to 9-month-old female rats (Fig. 1b). The density of NA nerve fibers increased significantly in all the compartments of thymuses from 24- and 31-month-old female rats, but especially in the cortex and paracortex (Figs. 1c and 1d). These age-associated changes in thymic morphology were accompanied by increase in the density of cortical autofluorescent cells present among the dense plexuses of NA nerve fibers (shown Fig. 1b).

ANOVA revealed significant differences in NE concentration and total NE content in the thymus (expressed in pmoles/mg protein; Fig. 2A and pmoles/mg wet weight; Fig. 2B, respectively) between the treatment groups. Post-hoc analysis demonstrated that NE concentration increased significantly ($P < 0.0005$) in the thymuses of early middle-aged and old female rats compared with the young female rats. The increase in NE concentration in old female rats were significantly ($P < 0.0005$) higher compared to young and middle-aged rats. No significant differences in thymic NE content (Fig. 2B) were observed between the two groups of old rats, or the middle-aged and old rats.

3.2. Age-associated decline in NA innervation in the spleen

Glyoxylic method of fluorescence histochemistry revealed dense NA innervation in various compartments of the spleen of young female rats. Thick plexuses of NA nerve fibers coursed in association with splenic artery entered the spleen and continued as vascular plexuses or along the trabeculae forming trabecular plexuses in the red pulp of the spleen (data not shown). In the white pulps of the splenic hilar region, NA innervation is abundant around the central arterioles with small linear and punctuate fluorescent profiles projecting into the

periarteriolar lymphatic sheath (PALS), a region rich in T lymphocytes (Fig. 3a). Bundles of NA nerve fibers were also present in the marginal zone, an area of densely packed macrophages and B lymphocytes. The pattern of NA innervation in the spleens from middle-aged female rats was similar to the profile observed in young female rats (Fig. 3b). In old rats, irrespective of the age groups, there was a decline in NA nerve fibers in all the compartments of the spleen compared with young rats (Figs. 3c and 3d). The density and the intensity of the fluorescent NA nerve fibers around the central arterioles and other vascular compartments were significantly reduced. The loss in NA innervation in spleens from old female rats was associated with diminished white pulp volume and an increase in the infiltration of yellow autofluorescent cells.

In the young and early middle-aged female rats, ICC for TH+ nerve fibers demonstrated bundles of nerve fibers traveling along with the splenic artery entering the spleen and continuing into the parenchyma of the spleen either as vascular plexuses or trabecular/capsular plexuses. The majority of TH+ nerve fibers coursed along the central arteriole of the white pulps from which numerous fine punctate nerve fibers extended into the PALS (young, Figs. 4a and early middle-aged, Fig. 4b). In addition, linear and punctate TH+ nerve fibers are present along the marginal sinus, parafollicular zones, and in the marginal zone. In contrast to young and early middle-aged female rats, ICC for TH revealed a drastic loss of nerve fibers around the central arteriole in the PALS, near the marginal sinus, in the parafollicular zone and in the venous and trabecular plexuses of the spleens from old female rats (Figs. 4c and 4d). There was also an accumulation of unstained golden granular cells in the white pulp of spleens from the early middle-aged and old female rats. Negative control sections confirmed the specificity of the staining for TH (data not shown)

Splenic NE concentration (pmoles/mg protein) were comparable in young and early middle-aged female rats, but was significantly ($P<0.001$) reduced in the hilar (Fig. 5A) and end regions (Fig. 5B) of the spleens of old rats irrespective of the old age groups compared with the young and early middle-aged female rats. In the hilar (Fig. 5C) region, total splenic NE content (pmoles/mg wet weight) was lower ($P<0.0005$) in old female rats compared with young and middle-aged female rats. In end region of the spleen (Fig. 5D), a similar decline in total NE content was observed in old compared with young female rats, but there also was a significant decline in early middle aged compared with young rats. Similarly, there was a significant ($P<0.0005$) age-related decline in the total NE content in the whole spleens (Fig. 5E) of early middle-aged and old female rats compared with young rats.

3.3. Age-associated decline in NA innervation in the MLN

Fluorescence histochemistry revealed dense plexuses of NA nerve fibers entering the hilus along with the blood vessels, and distributing into the subcapsule or along the vasculature coursing through the medullary cords in the MLN of young female rats (data not shown). These nerve fibers course into the medulla together with vascular and lymphatic channels and enter the paracortical regions that are rich in T lymphocytes (Fig. 6a), and continue as single varicose profiles into the cortical parenchyma. Compared with staining in young rats, there was an age-associated reduction in density of NA nerve fibers in all the compartments of lymph nodes, including the paracortical regions of the middle-aged (Fig. 6b) and old (Figs. 6c and 6d) female rats. Similar to the spleen, there was an increased localization of yellow autofluorescent cells in the MLN of middle-aged and old female rats. Reflecting the age-related loss of NA innervation in the lymph nodes, NE concentration (pmoles/mg protein; 7A) and content (pmoles/mg wet weight; 7B) also significantly ($P<0.01$) declined in the MLN of middle-aged and old female rats in comparison to young female rats.

3.3. Age-associated decline in splenic NK cell activity, T and B lymphocyte proliferation, IL-2 production, and IFN- γ production

NK cell activity for each age group and E:T ratio is shown in Fig. 8. The percent lytic activity was significantly reduced ($P<0.05$) at 80:1 and 40:1 E:T ratios in early middle-aged compared with young female rats. NK cell killing was dramatically lower ($P<0.0005$) at all but the lowest E:T ratio in both groups of old rats compared with young and early middle-aged groups.

Con A-induced T lymphocytes proliferative response progressively declined ($P<0.0005$) across age (Fig. 9A). There was a significant ($P<0.0005$) decrease in T cell proliferation in middle-aged 24-month-old, and 31-month-old rats compared with young female rats at each concentration examined (Fig. 9A). LPS-induced proliferation of B lymphocytes (Fig. 9B) was also significantly ($P<0.05$) reduced in old female rats in comparison to young female rats. Measurement of IL-2 (Fig. 10A) and IFN- γ (Fig. 10B) production was performed using supernatants obtained from Con A-stimulated lymphocytes from the spleen at a concentration of 1.25 $\mu\text{g/ml}$ Con A. IL-2 production was significantly ($P<0.0005$) lower in early middle-aged and 24-month-old female rats compared with young rats. It was further reduced ($P<0.0005$) in 31-month-old rats compared with young and early middle-aged female rats. IFN- γ production significantly ($P<0.0005$) declined in old female rats compared with young and early middle-aged female rats.

4. Discussion

Immunosuppression is a characteristic feature of the aging process, leading to increased risk for autoimmunity, cancer and infectious diseases. The results from the present study demonstrate that the reduction in immune responses in aged female rats are associated with a decline in sympathetic NA innervation in the secondary lymphoid organs, spleen and MLN, and an apparent increase in NA innervation in the thymus due to age-associated thymic involution. These age-related alterations in sympathetic NA innervation begin to appear in the early middle-aged rats evident by lower NE levels in the end region of the spleen similar to old female rats while NE levels in the hilar region is comparable to young rats. In addition, splenic Con A-stimulated IFN- γ levels did not show an age-related reduction but IL-2 production was lower in the early middle-aged female rats. The age-associated decline in sympathetic NA innervation in the spleen and mesenteric lymph nodes begins in middle-aged (8- to 9-month-old) and is more pronounced in old female rats compared to young female rats. Taken together, these findings suggest that there is an age-related alteration in the sympathetic NA regulation of immune response in thymus, spleen, and lymph nodes of female rats with profound changes in innervation and immune function apparent by early middle-age. Age-related changes in the cross-talk between the SNS and immune system may be contributory to the rising risk for age-related diseases such as autoimmunity and cancer, and for gender differences in risk for autoimmunity.

There are gender-based similarities and differences in the pattern of sympathetic NA innervation of lymphoid organs between male and female F344 rats (Table 1). The pattern and volume density of sympathetic NA innervation of the thymus were similar to that described in F344 male rats and female Lewis rats across age (Bellinger et al., 1988; Kranz et al., 1997). Sympathetic NA innervation in the parenchyma of the thymus of young female rats on the day of diestrus II, and in young male rats, was sparse, increased slightly in the early middle-aged female rats, but was very dense in the old female rats. Based on the estimated total thymic NE content we attribute the increased density of NA nerves in the aged thymus to reduce tissue volume in the absence of a change in number of NA nerves supplying the thymus. Whether, NA nerves play a role in thymic involution, which results from decrease in the cortex, is not known, there are only a few studies that have examined

the effect of NA innervation of thymocyte development in aging (Madden and Felten, 2001b).

It is also possible that age-related thymic involution is attributable to changes in the circulating levels of gonadal steroids observed during regularly cycling young rodents because the proportions of epithelial and cortical cells are higher during the estrus stage compared with diestrus stage in young rats and E administration to ovariectomized rats decreases thymocyte number with a concomitant increase in epithelial parenchyma (Glucksmann and Cherry, 1968). It is yet to be determined whether the thymocytes exposed to higher concentrations of NE due to thymic atrophy during aging leads to altered functional capacity of thymocytes observed in elderly. In adult male rats, β -adrenergic receptors are fewer in unfractionated thymocytes, whereas mature T cells in the thymus express the receptors at levels comparable to those seen on peripheral T cells (Madden and Felten, 2001b). Even though the receptors are expressed at low levels, these unfractionated thymocytes are capable of eliciting an isoproterenol-induced cAMP response that is equivalent to that of splenocytes where the receptor density is higher, suggesting that the thymocytes are sensitive to β -adrenergic receptor signaling (Madden and Felten, 2001b). Although the presence of β -adrenergic receptors on thymocytes of female mice has been established (Segal and Ingbar, 1980), further studies are essential to understanding the role of the SNS in modulating the differentiation and maturation of lymphoid cells during ontogeny and aging, and to determine the role of gender on SNS regulation. The age-related decline in T cell differentiation in the thymus with concomitant reduction in naïve T cell production is attributed to the age-related dysregulation of the thymic microenvironment, and results in reduced cell-mediated immunity, increased autoantibody production, and increased susceptibility to infectious disease (Thoman and Weigle, 1989). However, factors outside that immune system can also be contributory, since implantation of old female rats with pituitary adenoma cells secreting growth hormone and PRL or treatment of old female mice with growth hormone secretagogue restores the thymus with cortical thymocytes and medullary epithelial cells and, increases mitogen-induced splenic T lymphocyte proliferative responses and T helper cells (Kelley et al., 1986; Koo et al., 2001). Similar to growth hormone effects on the thymus, administration of insulin-like growth factor-I (IGF-I) to old mice increases thymic cellularity without affecting the thymic CD4⁺/CD8⁺ population (Montecino-Rodriguez et al., 1998). In contrast, introduction of IL-7-secreting stromal cells into the thymus of old female rats enhances the CD25⁺ cells but does not reverse thymic involution (Phillips et al., 2004). Therefore, the restoration of thymopoiesis and reversal of thymic involution in aged rodents requires both the hematopoietic molecules and endocrine factors in order to abrogate immunosenescence.

The age-associated reduction in sympathetic NA nerve density, and NE concentration and content, in the spleens of old female rats is consistent with the loss of sympathetic NA innervation observed in old male rats previously reported from our laboratory (Felten et al., 1987; Bellinger et al., 1992; Bellinger et al., 2008; Perez et al., 2009), but occurs at an early age than reported for male rats (Table 1). The age-related decline in NE levels was approximately 60 to 70% in all the splenic compartments of old female rats, which is similar to that observed in old male rats (Felten et al., 1987; Bellinger et al., 1992). NA innervation and NE levels in the spleen of middle-aged female rats decreases in the end region of the spleen, an area that is distal from the hilar region the entry site for NA nerves into the spleen, which also is similar to reports in male rats. These findings suggest sympathetic peripheral neuropathy (progressive distal-to-proximal “dying-back” of nerve fibers) beginning during early middle-age similar to the pattern of NA nerve loss in the spleen after treatment with the neurotoxin, 6-hydroxydopamine (Lorton et al., 1990).

The mechanism responsible for the more rapid decline in sympathetic innervation of secondary lymphoid organs in female rats remains to be determined. However, studies investigating interactions between female sex hormones and sympathetic regulation of reproductive functions support a possible role for estrogen in regulating sympathetic nerve density and activity in lymphoid tissue (Zoubina et al., 2000, 2001; reviewed in Brauer, 2008). Our findings of reduced innervation and NE concentration in secondary lymphoid organs during diestrus are consistent with hormonal effects on sympathetic nerves, but not with the direction of change since the density of myometrial sympathetic nerves is reduced with elevated levels of estrogen, and increased when estrogen levels are lowered. In the uterus, sex hormones cause a shift in the balance between signals with positive and negative effects on sympathetic nerve outgrowth, including pro-neurotrophins and neurotrophins (Chalar et al., 2003; Krizsan-Agbas et al., 2003; Chávez-Genaro et al., 2006; Varol et al., 2000; Lobos et al., 2005; neurotrophin receptor expression in sympathetic neurons (Richeri et al., 2005), and semaphorins (Richeri et al., 2007; Marzioni et al., 2004). Based on preliminary unpublished findings in our laboratory, we propose a similar role for estrogens in secondary lymphoid organs of the aging female rat.

Whether changes in NE levels during early middle-age indicate alterations in NE release/turnover in the spleen of female rats is unknown. Studies conducted in the spleens of male rats have demonstrated an increase in the NE turnover in 10-month-old middle-aged rats concomitant with a decline in cAMP production, suggesting impairment in β -adrenergic receptor signal transduction in splenocytes (Bellinger et al., 2008). Whether, similar functional changes occur in SNS in spleens from aging, female rats needs to be investigated, as well as, the possible influence of reproductive cycle. Similar to the findings in spleens of middle-aged female rats, NE release is higher in hypothalamic regions of middle-aged female rats, such as the medial preoptic area and medial basal hypothalamus that regulate the reproductive aging process. These findings suggest profound changes in the both central and peripheral NA systems that may mediate three-way cross-talk between the SNS, neuroendocrine system and the immune system to affect age-related changes in host defense that begin during middle-age (Wilkes et al., 1979; MohanKumar et al., 1994; ThyagaRajan et al., 1995). The notion that the age-associated decline in the growth factors and antioxidant enzyme activities in the spleens of female rats may facilitate the loss of sympathetic NA activity in the spleen resulting in immunosenescence needs to be investigated further (Gatzinsky et al., 2004; Bolzan et al., 1995).

Our results demonstrating an age-related reduction in the immune responses, including mitogen-induced proliferation and cytokine production, and NK cell activity in spleens from female rats are in accordance with other studies on aging in female rodents (Davila and Kelley, 1988; Simioni et al., 2007). Consistent with the less robust signs of sympathetic nerve loss in the early middle-aged compared with old female rats, significant age-associated reduction in immune responses was not evident in all measures assessed (only for IL-2 production and T cell proliferation), suggesting that profound changes in the population of T lymphocytes occurring in the spleen during middle-age that may then influence the ability to subsets of T lymphocytes to produce T helper (Th)1 vs. Th2 cytokines (Barrat et al., 1997). An absolute link between age-associated decline in sympathetic NA innervation in the secondary lymphoid organs and immunosenescence is yet to be established but the available scientific literature indicates that SNS has important regulatory functions over immune system and that this cross-talk modulates the immune homeostasis.

Hormonal fluctuations, especially E and P, during each estrous cycle alter immune responses. T and B cell proliferation and plaque-forming responses are suppressed during diestrus when the levels of E are low, but are enhanced during proestrus stage, which is characterized by high levels of E (Krzych et al., 1978). E has multiple effects on immunity

by differentially binding to different types of E receptors, including the nuclear receptors, estrogen receptor (ER)– α and ER– β , but their specific roles in modulating cell-mediated and humoral immune responses need to be explored at different stages of the estrous cycle across age (Kawashima et al., 1992; Islander et al., 2003).

The pattern of age-related loss of sympathetic NA innervation in MLN from female rats is similar to that observed in lymph nodes from young and aged male rodents taken from several body sites (Felten et al., 1984; Bellinger et al., 2001b). MLN are a key regulator of mucosal immunity by inducing tolerance against soluble proteins and serving as a guard against commensal organisms (Macpherson and Smith, 2006). Whether altered sympathetic neural-immune interactions induced by diminished sympathetic NA nerve fibers with aging leads to an age-associated decline in IgA antibody secretion is not known (Schmucker et al., 2003).

In summary, we have demonstrated that sympathetic NA innervation is altered in the primary and secondary lymphoid organs from female rats with advancing age, beginning at the early middle-age concomitantly with age-related immunosuppression. Advancing age in women increases the risk for certain types of autoimmune diseases, metastatic cancers associated with the reproductive tract and breasts, infectious diseases, Alzheimer's disease, and cardiovascular diseases (Dawson-Hughes, 2008; Tanriverdi et al., 2003; Anisimov et al., 2003; Curns et al., 2005; Genazzani et al., 2007; Goldfarb and Ben-Eliyahu, 2006; ThyagaRajan and Felten, 2002). Sympathetic NA innervation in secondary lymphoid organs is critical for maintaining the balance between Th1- and Th2-immune responses (Shearer, 1997). However, the modulatory effects of gonadal hormones, E and P, on the sympathetic NA neuronal activity and immunity need to be investigated to fully understand the mechanism(s) of altered neural-immune interactions in aged females.

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Figure 1. (a–d). Fluorescence histochemistry in the thymus from female F344 rats. Fluorescent NA varicosities surround blood vessels (large arrowhead) and a linear fluorescent profile (small arrowhead) is present in the cortex (c) of the thymus from a young 3-month-old female rat (Fig. 1a). Moderate to intense fluorescent plexus around the blood vessel (large arrowhead) and linear strands of fluorescent varicosities are seen in the thymic cortex near the corticomedullary junction of early middle-aged (Fig. 1b), 24- to 25-month old (Fig. 1c) and 30- to 31-month old (Fig. 1d) female rats. j, corticomedullary junction; m, medulla. Calibration bars = 50 μ m

Figure 2.

NE concentration and total content in the thymus (A and B) from female F344 rats. NE concentration (pmoles/mg protein; A) in the thymus was significantly ($P < 0.0005$) elevated in old compared with young female rats and early middle-aged rats while NE content (pmoles/mg wet weight; B) was higher ($P < 0.0005$) in the middle-aged and the two groups of old female rats. *Significantly ($P < 0.0005$) different from the young group. ** Significantly ($P < 0.0005$) different from young and early middle-aged groups.

Figure 3. (a–d). Fluorescence histochemistry in spleens from female F344 rats. A dense plexus of NA fibers (large arrowhead) surround the central arteriole (ca) and fluorescent varicosities (small arrowheads) are present in the splenic white pulp of young 3-month-old female rats (Fig. 3a). Moderate innervation of NA fibers is found along the central arteriole (ca) (large arrowhead) and in the parenchyma of the white pulp (small arrowheads) in the spleens of early middle-aged (Fig. 3b) while the NA innervation is sparse in 24- to 25-month old (Fig. 3c) and 30- to 31-month old (Fig. 3d) female rats. Calibration bars = 50 μ m.

Figure 4. (a–d). Single-label immunocytochemistry for TH+ NA nerve fibers in spleens from female F344 rats. A dense plexus of TH+ nerve fibers (large arrowhead) is seen along the central arteriole (ca) and in the adjoining cellular compartments of the white pulp (small arrowhead) in young 3-month-old female rats (Fig. 4a). The innervation is moderate around the central arteriole (ca) (large arrowhead) and in the splenic white pulp of middle-aged (Fig. 4b). In contrast to young and early middle-aged rats, there are very few TH+ nerves around the central arteriole (ca) and in the parenchyma (small arrowhead) of 24- to 25-month old (Fig. 4c) and 30- to 31-month old (Fig. 4d) female rats. Calibration bars = 50 μ m

Figure 5.

(A–E) NE concentration and content in spleens from female F344 rats. NE concentration (pmoles/mg protein) was significantly ($P < 0.001$) lower in the hilar (A) and end (B) regions of spleens from old rats compared with young and early middle-aged rats. NE content (pmoles/mg wet weight) was significantly ($P < 0.0005$) reduced in the hilar region (C) of the spleens of old rats compared with young and early middle-aged rats while it was significantly ($P < 0.0005$) lower in splenic end regions (D) of early middle-aged and old rats than in the young rats. NE content in the whole spleen (E) showed a significant ($P < 0.0005$) age-associated decline in early middle-aged and old rats compared with young rats.

Figure 6.

(a–d) Fluorescence histochemistry in MLN from female F344 rats. NA fibers (large arrowhead) course through the medullary region (m) associated with vascular plexuses and terminate as linear profiles (small arrowhead) among lymphocytes in the paracortical (p) regions of MLN of young 3-month-old female rats (Fig. 6a). Sparse innervation of NA fibers is found in the medullary region (m) and paracortical regions (p) (small arrowhead) of the MLN of early middle-aged (Fig. 6b), 24- to 25-month old (Fig. 6c) and 30- to 31-month old (Fig. 6d) female rats. Calibration bars = 50 μ m

Figure 7. Concentration and content in mesenteric lymph nodes (MLN; A and B) from female F344 rats. NE concentration (A) and content (B) in MLN was significantly ($P < 0.01$) reduced in early middle-aged and old rats than in young rats.

Figure 8.

Splenic NK cell activity in female F344 rats. NK cell activity was significantly ($P < 0.05$) reduced in the spleens of early middle-aged female rats relative to young rats at 80:1 and 40:1 ratios. The reduction in NK cell activity was more pronounced ($P < 0.0005$) in old female rats compared to early middle-aged and young female rats at 160:1, 80:1 and 40:1 ratios.

Figure 9.

Con A-induced T lymphocyte proliferation (A) by spleen cells from female F344 rats. Spleen cells were incubated with 0, 0.3, 1.25, or 5 μg/ml of Con A for 72 h. Proliferation of T lymphocytes was reduced in early middle-aged and old female rats compared with young rats. LPS-stimulated B lymphocyte (B) by spleen cells from female F344 rats. Purified splenocytes were incubated with medium or a single concentration of LPS (5 μg/ml) for 72 h. Similar to Con A-induced T lymphocyte proliferation, LPS-induced B lymphocyte proliferation was suppressed in old rats compared with young female rats. *Significantly ($P < 0.05$) different from young group. LPS-induced B lymphocyte proliferation was not measured in early middle-aged female rats.

Figure 10.

IL-2 production (A) by spleen cells from female F344 rats. Spleen cells were incubated with 1.25 $\mu\text{g/ml}$ of Con A for 24 h. Supernatants were cultured with the IL-2-dependent cell line, CTLL, and cell growth was measured colorimetrically. *Significantly ($P < 0.0005$) different from the young group. ** Significantly ($P < 0.0005$) different from young and early middle-aged groups. IFN- γ production (B) by spleen cells from female F344 rats. Spleen cells were incubated with 1.25 $\mu\text{g/ml}$ of Con A for 24 hrs. Supernatants were used for measurement of IFN- γ production by ELISA. *Significantly ($P < 0.0005$) different from young and early middle-aged groups.

Age-associated differences in the pattern of sympathetic NA innervation in the lymphoid organs of male and female F344 rats

Table 1

Lymphoid Organs	Thymus			Spleen			Mesenteric Lymph node		
	3	8-9	25	3	8-9	25	3	8-9	25
Age (months)									
Sex									
Male *	+	+++	++++	++++	++++	+	++++	++++	+
Female	+	+++	++++	++++	++	+	++++	+	+

* Based on Bellinger et al., 1988, 1992, and 2001b

+ Semi-quantitative evaluation of nerve density using a + to +++++ system where + and +++++ represent the lowest and highest density, respectively, and performed with raters blinded to the age of the rat.