ABSTRACT

Background

Homeostatic functions at the cellular, tissue, organ and the systemic level are maintained by bi-directional communication between the nervous system, the endocrine system and the immune system. Neuroendocrine outflow from the brain is transduced through the sympathetic nervous system and is tightly regulated by feedback loops. Neural-immune connection is established directly through sympathetic noradrenergic innervation of lymphoid organs and indirectly through immunomodulatory hormones from neuroendocrine output. Sympathetic innervation of lymphoid organs, direct synaptic association of lymphocytes with noradrenergic nerve fibers and the presence of adrenergic receptors on lymphocytes suggest that sympathetic stimulation may alter immune functions. Immune effector molecules in turn can bind to central targets and mediate systemic functions; for example, the role of cytokines in mediating sickness behavior.

In young age, the neuroendocrine-immune network is robust and is capable of tremendous plasticity at the same time is tightly regulated to achieve homeostasis in the face of stressful stimuli. However, with age, there is degeneration of nerve fibers, loss of compensatory mechanisms, decreased release of active neural, endocrine and immune mediators, compromised responsiveness to neural, endocrine and immune signals contribute to dysregulation of the neuroendocrine-immune network.
One among the key outcomes of dysregulation of the neuroendocrine-immune network is the decline in cell-mediated immune functions which predisposes the development of age-related diseases like autoimmunity and cancer.

Age-related decline in immune functions is largely attributed to the loss of sympathetic noradrenergic innervation of lymphoid organs although the actual effects of sympathetic stimulation on immune effector cells at the lymphoid milieu in the resting state have not been outlined yet. Throughout reproductive life, the neuroendocrine-immune milieu in females is influenced by rhythmic alterations in the levels of the female gonadal hormones especially estrogen and progesterone. The immunomodulatory role of estrogen has been widely shown in vivo although the direct effects of the hormone on cell mediated immune functions in stimulated lymphocytes and lymphocytes in the resting state have not been characterized yet. Evidence from our laboratory and others indicate that the onset of age-associated loss of sympathetic noradrenergic fibers in rodents is much earlier in females compared with males implicating the degenerative effects of precluding exposure to estrogen throughout reproductive life and in the perimenopausal period. This obviates the need to study the role of estrogen in modulating immune functions individually and in the presence of adrenergic agents.

Decline in neuroendocrine control of immune functions and the onset of cell-mediated immunosenescence preceeds the development of age-associated diseases such as autoimmunity and breast cancer in females. Strategies to reverse sympathetic denervation-associated decline in immune functions by using the synthetic monoamine oxidase-B inhibitor deprenyl, have shown to have beneficial effects in animal models of mammary tumorigenesis. Regeneration of sympathetic noradrenergic fibers in the lymphoid organs of rodents with DMBA-induced mammary tumors was associated with significantly enhanced cell-mediated immune functions and tumor regression. Several studies have explored the presence of adrenergic receptors on mammary tumor cells and have studied the effects of adrenergic stimulation on breast cancer cell proliferation and pro-angiogenic markers. However, considering the strong role of the endocrine component in lymphoid sympathetic denervation in females by estrogen and the implications of estrogen responsiveness on the incidence and progression of mammary tumors further studies are warranted to differentially understand how sympathetic agents modulate the proliferation
and pro-angiogenic secretions of tumor cells themselves. Since estrogen responsiveness is a crucial discriminating factor in mammary tumorigenesis, it is vital to understand the effects of adrenergic stimulation of estrogen receptor positive and negative tumor cells on these survival signals.

From a clinical perspective adrenergic signaling is a molecular translation of stress-associated sympathetic outflow. The knowledge of having a potential life-threatening disease like breast cancer and its associated emotional and psychological burden render stress-related signaling a crucial determinant of breast cancer progression and prognosis as shown in psychosocial support studies. Perception of emotional and financial support and involvement in support groups, meditation, and interventions such as guided imagery have been shown to exert beneficial influences on breast cancer patients. The psychological component can influence the functioning of the neuroendocrine-immune network and mediate beneficial effects probably through modulation of adrenergic signaling cascades. In the Indian context, practices such as spirituality and meditation play a powerful role as it is ingrained in the culture unlike the western population who are taught to adopt certain practices. Several studies have been published citing the beneficial effects of spirituality in influencing prognosis among patients with breast cancer. However, the molecular mechanisms behind the beneficial effects of spirituality on the neuroendocrine-immune network possibly through noradrenergic mechanisms warrants further studies.

**Objectives:**

Based on the gaps in research identified above, the following objectives have been identified:

1. To examine the direct dose-dependent and receptor subtype-specific effects of 17β-estradiol and estrogen-receptor (ERα- and ERβ-) agonists and antagonists on splenic lymphocyte functions (cytokine production (IL-2, IFN-γ), compensatory mechanisms (antioxidant enzymes, nitric oxide production) and molecular markers (p-ERK, p-CREB, p-Akt) in vitro using splenocytes stimulated with Con A and in the resting state

2. To examine in vitro estrogen-induced modulation of the differential effects of adrenergic stimulation on resting lymphocyte functions such as cytokine production (IL-2, IFN-γ), compensatory mechanisms (antioxidant enzymes,
nitric oxide production) and molecular markers (p-ERK, p-CREB, p-Akt, p-NF-
κB) using α₁-AR, α₂-AR, β₁-AR and β₂-AR-specific agonists and antagonists in combination with or without 17β-estradiol.

3. To examine the role of receptor-specific adrenergic stimulation on hormone-responsive (ER+ MCF-7) and hormone non-responsive (ER- MDA MB-231) breast cancer cell line proliferation, pro-angiogenic molecule expression (VEGF A, VEGF C and NO), compensatory machinery (antioxidant enzymes) and molecular signaling mediators (p-ERK, p-CREB, p-Akt) in vitro.

4. To examine the correlative effects of spirituality in modulating immune functions in vitro on Con A-stimulated and LPS-stimulated peripheral blood mononuclear cell proliferation, signaling molecule expression (p-ERK, p-CREB and p-Akt), compensatory mechanisms (antioxidant enzymes), and psychometric assessment of physical, emotional and functional well-being in India women with breast cancer.

Methods

Briefly, lymphocytes were isolated from the spleen of male Sprague-Dawley rats using density gradient centrifugation and treated in vitro with 17β-estradiol(10⁻¹⁴ M, 10⁻¹² M, 10⁻¹⁰ M, 10⁻⁸ M, 10⁻⁶ M) or estrogen receptor agonists ERα-specific: PPT (10⁻¹⁰ M, 10⁻⁸ M, 10⁻⁶ M) or ERβ-specific DPN (10⁻¹⁰ M, 10⁻⁸ M, 10⁻⁶ M) with or without ER antagonists ICI(182,780) (10⁻⁵ M) or tamoxifen (10⁻⁵ M) and their effects on resting/ con A (1.25 µg/ml)-induced splenocyte functions were assessed as cited in the objectives. In another experiment, lymphocytes were co-incubated with receptor subtype-specific adrenergic agonists and antagonists (α₁-AR: phenylephrine (10⁻⁹ M, 10⁻⁶ M, 10⁻³ M) and prazosin (10⁻⁵ M); α₂-AR: clonidine (10⁻⁹ M, 10⁻⁶ M, 10⁻³ M) and idazoxan (10⁻⁵ M); β₁-AR: dobutamine (10⁻⁹ M, 10⁻⁶ M, 10⁻³ M) and metoprolol (10⁻⁵ M) and β₂-AR: terbutaline (10⁻⁹ M, 10⁻⁶ M, 10⁻³ M) and propranolol (10⁻⁵ M)) with or without 17β-estradiol (10⁻⁹ M) in order to establish the dose and receptor sub-type dependent effects of estrogen and adrenergic agents on splenocytes.

Breast cancer cell lines MCF-7 and MDA MB-231 on the other hand were cultured independently in DMEM and L-15 medium respectively supplemented with 2mM L-glutamine, 7.5% sodium bicarbonate and 10% FBS. The cells were co-incubated with
receptor subtype-specific adrenergic agonists and antagonists ($\alpha_1$-AR: phenylephrine ($10^{-9}$ M, $10^{-6}$ M, $10^{-3}$ M) and prazosin ($10^{-5}$ M); $\alpha_2$-AR: clonidine ($10^{-9}$ M, $10^{-6}$ M, $10^{-3}$ M) and idazoxan ($10^{-5}$ M); $\beta_1$-AR: dobutamine ($10^{-9}$ M, $10^{-6}$ M, $10^{-3}$ M) and metoprolol ($10^{-5}$ M) and $\beta_2$-AR: terbutaline ($10^{-9}$ M, $10^{-6}$ M, $10^{-3}$ M) and propranolol ($10^{-5}$ M)) in order to establish the dose and receptor sub-type dependent effects of adrenergic agents on ER+ and ER- breast cancer cell lines.

PBMCs were isolated from spiritual and non-spiritual young, middle-aged women and breast cancer patients using density gradient centrifugation and stimulated with 0.5, 1.25 and 5 µg/ml concanavlin A (Con A) or 0.01, 0.1 and 1 µg/ml lipopolysaccharide (LPS) for 72 hours. FACIT-B questionnaire was collected from all the participants and the spirituality score was ascertained. Linear regression analysis was performed to correlate spirituality scores with immune functions, physical, emotional and functional well-being to assess the role of spirituality in affecting women with breast cancer.

**Results**

Estrogen enhances lymphoproliferation and IFN-γ production in a dose-dependent and receptor sub-type specific manner. Estrogen receptor specific agonists differentially induce IL-2 production in vitro. Estrogen treatment increased the phosphorylation of intracellular molecular markers such as p-ERK, p-CREB, and p-Akt and enhanced the activities of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase-isoform 1 (GPx-1). Estrogen and its agonists differentially regulate nitric oxide production to affect immunity.

Adrenergic agonists and antagonists were immunosuppressive in a receptor-independent manner. $\alpha_2$-AR agonist Clonidine suppressed lymphocyte proliferation, enhances nitric oxide production and NFkB phosphorylation through an iNOS-dependent mechanism. On the other hand $\alpha_1$-AR agonist phenylephrine suppressed lymphocyte proliferation in an iNOS-independent mechanism. $\beta_2$-AR agonist terbutaline suppressed proliferation but enhanced IL-2 and IFN-γ production in a PKA and ERK-dependent pathway. Immune-enhancing effects of estrogen on adrenergic immunosuppression were dominant. Estrogen either alone or through β-AR differentially modulates immunity through ERK, PKA, and PKC pathways and NO.
Adrenergic agonists and antagonists selectively modulate the proliferation, VEGF A and C production and nitric oxide production of ER+ and ER- breast cancer cell lines by altering the molecular signaling pathways such as ERK, CREB and AKT.

In the clinical study, the spirituality scores from the psychometric analysis positively correlated with Con A-induced proliferation of PBMCs, physical well-being, emotional well-being and functional well-being in women with breast cancer. Thus, spirituality can exert beneficial effects in breast cancer by improving Con A-induced T-cell proliferation in spiritual women with breast cancer compared with their non-spiritual counterparts through specific molecular signaling pathways such as p-ERK, p-CREB and p-Akt.

**Conclusions**

Taken together, these results suggest that reproductive aging and development of diseases and cancer in females may be due to diverse effects of estrogen-induced regulation of adrenergic receptor actions on sympathetic modulation of immunity through specific intracellular signaling pathways assisted by compensatory factors such as antioxidant enzyme activities and growth factor biosynthesis. Further studies have to be carried out to clarify the role of estrogen in the loss of fibers in the periphery and to explore receptor transactivation and cross-talk mechanisms that may play a key role in vivo in order to better understand the implications of AR activation in mammary tumor progression.