CHAPTER 1

INTRODUCTION

The nervous system, endocrine system, and immune system function as an intricate interdependent network and their mutual interactions maintain physiological homeostasis [1]. The nervous system modulates endocrine functions through the release of neurotransmitters, growth factors and neuropeptides that can trigger the release of trophic hormones from the pituitary. These trophic hormones in turn influence target glands such as the gonads, to mediate the release of hormones such as estrogen that mediate systemic effects. The endocrine system exerts regulatory control over the nervous system through hormone-mediated feed-back inhibition of the hypothalamic neurons involved in the release trophic hormones. Also, endocrine secretions modulate immune functions centrally and systemically and are implicated in the development of autoimmune diseases and gender-related differences in immune functions [2,3]. Neuroendocrine control of immune functions are best noted in the immunosuppressive effects of glucocorticoids which have been widely studied in stress and disease. The implications of the female steroid hormone estrogen in the prevalence and pathogenesis of autoimmune diseases also indicate endocrine-regulation of immune functions [3-6].

In addition to these indirect effects on immune functions, the nervous system can directly influence immune functions through synaptic associations of sympathetic
noradrenergic nerve fibers on lymphocytes in the bone marrow, thymus, spleen, lymph nodes and other lymphoid tissues. The immune effector cells (T-cells and B-cells) express a host of adrenergic receptors to bind the norepinephrine released by these nerves which activates receptor-subtype-specific down-stream signaling pathways and mediate immunomodulation. The neural-immune network, like the neuroendocrine network is bidirectional. Neuronal cells also possess specific receptors that can bind immune effector molecules (cytokines) and target derived growth factors secreted by immune cells in the periphery [1]. Studies on sickness behavior show that centrally, immune effector molecules such as cytokines can cross the blood-brain barrier and modulate neural functions and psychosocial factors have been shown to influence survival in terminally ill patients and metastatic recurrences in breast cancer patients through altered immune functions [7-10]. Thus, neuroendocrine outflow from the brain via the pituitary or through direct sympathetic noradrenergic activity can influence immune functions in the periphery. However, the functional role of these fibers in the maintenance of immune homeostasis has not been explored yet.

Aging is marked by a general decline in the neuroendocrine-immune network characterized by reduced sympathetic activity, decreased responsiveness to catecholamines, decline in hormonal secretions and responsiveness and increased reactive oxygen species load. Of crucial significance is the age-associated loss of sympathetic noradrenergic fibers in the peripheral lymphoid organs and the concomitant decline in immune responsiveness. All of these factors contribute to deregulation of the synchronous functions of the neuroendocrine-immune network and the ensuing loss of homeostasis favors the development of age-associated diseases and cancer [1-3]. This is more profound in the case of females where a precipitous decline in estrogen levels accompanies altered immune functions and incidence of autoimmune diseases and hormone-dependent cancers [3-6]. Although the physiological relevance of sympathetic noradrenergic innervation of lymphoid organs is yet to be defined, age-associated loss of innervation has been found to be associated with immune senescence and favors the development of age-related diseases like cancer. Restoration of sympathetic noradrenergic nerve fibers in the lymphoid organs of aged rodents using the monoamine oxidase inhibitor deprenyl was associated with improved cell-mediated and innate
immune functions. Another interesting phenomenon is the earlier onset of sympathetic denervation in female rodents compared with male which may implicate the involvement of the female steroid hormone estrogen.

Reproductive life in female rats is characterized by rhythmic fluctuations in estrogen levels beginning from the onset of puberty at 35-40 days. The regular 4-day estrous cycles continue through their adult life until a period of irregular estrous cycles begin 8-10 months. This period is followed by a constant estrus phase at 10- to 19-months finally leading to cessation of reproductive cycles. This phase is characterized by disruption of regular endocrine rhythms by a constant estrus period, followed by persistent diestrus and anestrus stages by 19- to 30-months of age [3,4]. Exposure to increased levels of estrogen during the pro-estrous and perimenopausal periods have been shown to affect immune functions. The regular fluctuations observed in circulating levels of estrogen during the estrous cycle influence the proliferation of T and B cell populations and alter the localization of IgA-producing plasma cells [11]. Further, the ability of estrogen to shift the Th1/Th2 cytokine balance in favor of Th2 cytokines and promote humoral immunity has been implicated in the predominant incidence of autoimmune diseases in women [11-14]. The immunomodulatory effects of estrogen have been studied by our laboratory and others in young, middle-aged perimenopausal and old post-menopausal women. Interestingly the characteristic changes in hormone rhythms during the menstrual cycle are associated with alterations in immune functions during the follicular and luteal phases in young women suggesting a concentration-dependent role of estrogen in modulating immune functions. Also, age-associated loss of cell-mediated immune functions correlated positively with decline in estrogen levels and loss of compensatory machinery such as antioxidant enzyme activities and expression of target-derived growth factors [15].

*Although several studies have associated preferential shift to pro-inflammatory immune responses with estrogen levels, the direct effects of estrogen on lymphocytes in a dose-dependent manner have not been studied yet.*
The rhythmic secretion of estrogen and progesterone throughout the female reproductive cycle and its precipitous decline with reproductive senescence contributes to physiological and immunological changes that are implicated in the development of a plethora of age-associated female-specific diseases such as mammary tumors and autoimmune diseases [15-20]. Loss of central hypothalamic dopaminergic activity, resulting in increased prolactin secretion and denervation of sympathetic noradrenergic nerve fibers in the peripheral lymphoid organs with age precedes the loss of estrogen, decline in T-cell responses and incidence of mammary tumors in female rodents [6,21,22]. Estrogen mediates its immunomodulatory effects by binding to specific estrogen receptors ERα and ERβ and activating receptor subtype-specific down-stream signaling molecules. In rodents with and without autoimmune diseases, estrogen treatment leads to enhanced Th1 cytokines and proliferation of CD4+ T cells that have been shown to involve ERα-associated mechanism [23, 24].

**Although both ERα and ERβ are expressed on lymphocytes, the contribution of these receptors to estrogen-mediated alterations in cell-mediated immune functions is unexplored.**

Differences in receptor densities on T and B cells however indicate that ERα may regulate T cell maturation and differentiation in the thymus while ERβ may regulate B lymphopoiesis in bone marrow and determine their capacity to produce cytokines and antibodies [25,26]. One other mechanism is by direct translocation into the nucleus and binding with ERE, transcription factors like NF-kB, AP1 or ERK. However, the mechanistic implications of receptor-specific or concentration-dependent activation and ensuing signaling cascades have not been preferentially or differentially assessed [26].

Studies from our laboratory and others have established that immune functions are regulated by neuroendocrine outflow from the brain and direct sympathetic noradrenergic innervation of the lymphoid organs that form synaptic associations with immune cells [27, 28]. Norepinephrine released by sympathetic noradrenergic (NA) nerve fibers binds to specific receptors on the lymphocytes in the primary and secondary lymphoid organs and influence immune responses [6, 27, 28]. Similarly, soluble immune mediators such as
cytokines influence the functions of the central nervous system by crossing the blood-brain barrier and binding to specific receptors on neurons to influence mood, behavior and cognitive functions [27-29]. Differential distribution of adrenergic receptors has been observed in the lymphoid organs that exhibit different affinities to bind and transduce norepinephrine-mediated signals to alter the immune responses [28-30]. While β2-adrenergic receptors predominate the lymphoid cells of rodents and humans, the expression of α1- and α2-receptors on natural killer (NK) cells and T and B lymphocytes is hormone or cytokine-induced [29-33]. Activation of AR α1- and α2-mediated signal cascades by norepinephrine has been shown to influence lymphocyte proliferation, T helper (Th1 and Th2) cytokine production, apoptosis of lymphocytes and increase IgM antibody production [33-36]. However, little is known about the role of α1- and α2-AR in altering immune reactivity especially, in the presence of estrogen although centrally they are known to modulate the female reproductive behavior, lordosis [37]. It is possible that estrogen-induced effects on proinflammatory cytokines such as TNF-α in peritoneal macrophages may be mediated through α2-AR in the peripheral nervous system to affect cell-mediated immune responses [38].

Activation of norepinephrine mediated signals via the β2- receptors is dependent upon type of activation, time of engagement, and expression of co-stimulatory molecules such as CD86 [29,39,40]. Terbutaline modulates lymphocyte cytokine production in a cell-type dependent manner due to variations in the receptor density in immune cell subsets and responsiveness to trigger intracellular signaling molecules [41, 42]. β2-AR signals are transduced through several intracellular signaling pathways that involve PKA, PKC, Akt, NF-κB and various other molecular targets [39]. The immunomodulatory role of β2-adrenoceptors have been extensively researched although how estrogen affects β2-AR signaling in lymphocytes remains uninvestigated. Understanding the interaction between NE through specific adrenergic receptors and estrogen in females is critical to unraveling the mechanisms of the cross-talk neuroendocrine system and immune system in women's health and diseases.
Dysregulation of the neuroendocrine-immune network in female reproductive aging is characterized by central and peripheral denervation coupled with increased allostatic load, compromised cell-mediated and innate-immune functions resulting in susceptibility to autoimmune diseases, degenerative diseases like osteoporosis and mammary cancer[1,43,44]. Initiation and progression of mammary tumors involves several complex interactions and contributions between the neuroendocrine and immune mediators[2, 45-47]. The endocrine component plays a crucial role according to the neuroendocrine theory of mammary tumorigenesis that says that age and repetitive exposure to proestrous surges of estrogen impairs tuberoinfundibular dopaminergic activity in the hypothalamus, leading to increased prolactin (PRL) secretion which in turn contributes to tumor development [2, 48-50]. Carcinogen-induced mammary tumor models have illustrated the regulatory roles of the hypothalamus, the anterior pituitary and the ovaries in the development and growth of tumors where their products (prolactin (PRL) and estrogen (E)) mediate cooperative or independent roles in hormone-sensitive tumor progression [2,16].

*In vivo* studies have shown that prolonged treatment with Estrogen significantly enhances the rate of incidence of mammary tumors in rats owing to its potent mitogenic action on female mammary cells, thereby acting as a major stimulus for the growth of hormone-dependent cancers by binding E receptors located in the cell nucleus [51]. On the other hand a significantly decreased probability of breast cancer incidence and increased induction of breast cancer remission is observed in ovariectomized women substantiating the role of estrogen in mediating incidence and progression of the disease [2, 51]. Interestingly, women who underwent early pregnancies have shown to have significantly reduced risk of breast cancer against pregnancies beyond the age of 35 years [2, 52]. Antiestrogen endocrine therapy using selective estrogen receptor modulators such as tamoxifen, antiprogestational agents such as mifepristone and luteinizing hormone analogues are popular treatment approaches for perimenopausal and postmenopausal women with breast cancer favored more than chemotherapy although there is a risk of aggressive recurrence and cervical cancers [44].
Although estrogen is thought to be involved in mediating peripheral degeneration of sympathetic noradrenergic fibers in the lymphoid organs as well, further studies are required to link estrogen with peripheral denervation, ensuing immunosenescence and mammary tumorigenesis causally.

An equally compelling role is played by the age-related sympathetic noradrenergic denervation of lymphoid organs, suppression of cell-mediated immunocompetence and the ensuing imbalance in the production of T helper 1 (Th1) cytokines, IL-2 and IFN-γ along with a host of related factors forming the basis for the immunological theory of mammary carcinogenesis [6, 53-54]. Anti-tumor immune defense involves both specific and non-specific immune effectors such as natural killer cells (NK cells), macrophages, polymorphonuclear cells, lymphokine activated killer cells (LAK cells), as well as various cytokines; and cytotoxic T-lymphocytes (CD8), CD4 T cells, and B cells that via antibodies direct cell-mediated cytotoxicity [55]. Macrophages and NK perform tumor surveillance in an activation-independent manner for preventing metastasis [56, 57]. Some of the cytokines secreted by lymphoid (macrophages, NK cells, T- and B-cells) and non-lymphoid (endothelial cells and fibroblasts) cells show anti-tumor activity such as IL-2 and IFN-γ [58-61]. On the contrary, cytokines like Interleukin-8 (IL-8) are secreted by various types of cancer cells as a tumor-secreted autocrine growth factor capable of inducing the expression of VEGF and mediate angiogenesis through the PKC pathway [62]. Immumomodulation by neural effector molecules such as catecholamines and neuropeptides are widely characterized centrally and peripherally in healthy aging and cancer through altered functions of NK cell, T cell, and B cell activities, including cytokine production, lymphocyte proliferation, cytotoxicity, and antibody production [28, 63, 64]. Peripherally, these molecules are released by the sympathetic noradrenergic and peptidergic innervation of primary and secondary lymphoid organs and also adrenal medullary catecholamines in circulation [28, 63, 64].

Developing a comprehensive understanding of adrenergic stimuli-mediated effects on cell-mediated immune effector cells and breast cancer cell lines will help better delineate the plethora of events that are triggered by stress and endocrine rhythms and facilitate the understanding of tumorigenesis and tumor progression.
Once carcinogenesis has been triggered, local progression and metastasis requires vigorous growth of neovascular tissue aided by the angiogenic factors such as vascular endothelial growth factor (VEGF), cytokines such as IL-8 and hormones such as estrogen. This process of angiogenesis is a crucial determinant of sustained neoplastic growth which is driven by pro-angiogenic signaling molecules expressed by the tumor cells themselves. The balance between pro-angiogenic factors and endogenous angiogenic inhibitors does not favor the activation and migration of the endothelium in normal tissues owing to the regulatory effects of tumor suppressor genes, oncogenes, adhesion molecules, factors promoting apoptosis, and the immune system [65]. However, in cancer cells the expression of pro-angiogenic molecules is enhanced to facilitate tumor vascularization in order to meet the demands of the rapidly proliferating cells for nutrient supply and sustenance. One of the crucial pro-angiogenic growth factors involved in the progression of breast cancer is the tumor-secreted multifunctional cytokine: vascular endothelial growth factor (VEGF). Apart from serving as a pro-angiogenic signaling molecule, VEGF also acts as an autocrine growth factor for the tumors and is involved in monocyte trafficking by inducing monocyte motility [66-68]. Adrenergic signaling is directly related to VEGF production in tumor cells because norepinephrine is a potent inducer of VEGF expression as seen in nasopharyngeal carcinoma cells and rat cardiac myocytes [69, 70].

Hence it would be interesting to study how receptor-specific activation of adrenergic receptors can differentially alter the expression of pro-angiogenic signaling molecules in hormone-responsive and non-responsive breast cancer cells and affect cellular proliferation and down-stream signaling molecules.

Functional relevance of adrenergic signaling in a clinical perspective among women with breast cancer can be drawn from the psychological toll of the disease on the patient. The psychological fear of having a deadly disease, environmental stressors, lack of social support and financial security, the stress of undergoing rigorous treatment regimen, fear of unfavorable prognosis or relapse are transduced through sympathetic stimuli. These stimuli can activate noradrenergic signals and modulate the disease progression through several mechanisms including immunosuppression and tumor
Women diagnosed with cancer have been shown to experience high levels of stress, anxiety, depression, and other psychiatric disorders [71,72]. Repeated exposure to stressful stimuli disrupts adaptation (allostasis) and causes accumulation of allostatic load resulting in altered immune reactivity and probably leading to development of various diseases including cancer [73].

Most of these effects are mediated by the release of catecholamines that exert their effects on lymphocytes mainly through the presynaptic α2 and postsynaptic β2 adrenergic receptors to affect the immune responses [70]. However, only few studies have provided initial evidence for the modulatory role of catecholamines in mammary cancer. Adrenergic receptors subtype, α2, are expressed on the human breast cancer cells and activation has been known to enhance proliferation of these cells and are also involved in mitogenic effect of certain catecholestrogens [74,75]. Treatment of mice with α2-adrenergic antagonists, yohimbine and rauwolscine, prevented the enhancement of tumor growth [76]. Recent studies also indicate that most of the breast, colon, and prostate carcinoma tissues express the relevant β2-adrenoceptors. Psychosocial stress observed in people with chronic disease conditions are often associated with increased pro-inflammatory markers such as IL-6 and C-reactive protein and decreased Th1 immune functions. Conversely, the balance is altered favoring better Th1 responses in cases where psychosocial support from friends, family, and society is perceived [9, 10].

There are several alternative therapeutic options built upon the benefits of psychosocial support and mind-body medicine that are known to benefit cancer patients such as meditation, Yoga, Pranic healing, Reikki, guided imagery, and spirituality. However, the scientific basis of these beneficial effects have not been widely explored. Majority of Indians, irrespective of their religious background, are spiritual in nature. Yogic exercises, meditation, chants and prayers are stringent daily rituals followed by many Indian women. This is unlike Western countries where such populations are in minority and are taught to adopt certain practices. Hence, it is relevant to study the effect of spirituality in modulating the neural-endocrine-immune interactions in mammary cancers in the context of Indian women. The effects of spirituality on the neuroendocrine-
immune network have been the focus of several studies indicating beneficial effects in breast cancer patients. However, the molecular signaling mechanisms through which the benefits of spirituality are transduced possibly involving norepinephrine and stress-associated modulations have not been explored yet in Indian women.

The present proposal is designed to bridge these gaps so that the results generated would be useful in identifying the neuroendocrine-immune biomarkers in breast cancer that might facilitate development of better therapy for women with breast cancer.

**Hypothesis:**

Our hypothesis is that altered homeostatic functioning of neuroendocrine-immune network especially; the neuroendocrine outflow to the sympathetic noradrenergic activity in the lymphoid organs results in immunosuppression and therefore, promotes mammary tumorigenesis. We believe that this derangement in the neuroendocrine-immune system is caused by the chronic effects of proestrous-surge in estrogen release resulting in the diminution of sympathetic NA activity to induce immunosuppression and subsequent development of mammary tumors. The scope of the present proposal is focused on testing our hypothesis that an enhancement of the neuroendocrine-immune interaction aided by suppression of angiogenic factors is important in arresting tumor growth. Adrenergic stimulation through norepinephrine or through trans-activating signaling molecules play a role in maintaining resting lymphocyte functions and derangement of this regulation during aging due to sympathetic noradrenergic denervation of lymphoid organs may contribute to the development of mammary tumors and other age-related female-specific diseases. Strategies that may help reverse sympathetic noradrenergic denervation or boost compensatory mechanisms and restore noradrenergic signaling such as neuroendocrine-immune modulators such as spirituality through psychosocial signals that may be transduced through norepinephrine may benefit women with breast cancer.
The following questions are designed to test our hypothesis:

(1) What is the role of estrogen in modulating neuroendocrine-immune network in the splenic lymphocytes?

(2) What is the extent of involvement of sympathetic NA neuronal activity in modulating proliferation, pro-angiogenic factors and signaling molecules in hormone sensitive and non-sensitive breast cancer cell lines?

(3) Does spirituality act as a neuroendocrine modulator in order to produce co-relative benefits in immune functions in patients with breast cancer?

Objectives:

On the basis of these questions the following specific objectives have been identified.

A. **Specific Objective 1:**

   Establish the Influence of Estrogen (E) and Adrenergic Receptor (AR) agents (agonists and antagonists) on modulation of splenic lymphocyte functions.

   The working hypothesis for this aim is that circulating estrogen will affect sympathetic-immune modulation to alter mammary tumorigenesis. The approach to test this hypothesis is to examine the effect of estrogen and estrogen receptor-specific agonists and antagonists on splenic lymphocyte functions. Subsequently assess the effects of (a) α₁- and α₂- adrenergic receptor and (b) β₂-adrenergic receptor agonists and antagonists, on splenic lymphocyte functions of male Sprague-Dawley rats in the presence and absence of hormonal stimulation with 17β-estradiol.

B. **Specific Objective 2:**

   Involvement of Cellular Mechanisms in AR-induced effects on ER (+) and ER (-) human breast cancer cell lines MCF-7 and MDA MB-231.

   Experiments were conducted to understand the role of AR on breast cancer cell growth and also, the role of intracellular signaling pathways in AR-induced alteration of human breast cancer cellular growth. Direct effects of adrenergic receptor agents on ER positive and negative human breast cancer cell lines were assessed in the modulation of
proliferation, production of pro-angiogenic factors and intracellular signaling pathways important for tumor growth.

C. **Specific Objective 3:**

**Correlative assessment of spirituality-induced effects on the neuroendocrine-immune network: Effects on cell-mediated and humoral-mediated immune responses**

Altered homeostatic functioning of neuroendocrine-immune network especially, the neuroendocrine outflow the sympathetic noradrenergic activity in the lymphoid organs results in immunosuppression and therefore, promotes mammary tumorigenesis. An enhancement of the neuroendocrine-immune interaction aided by psychosocial benefits of spirituality may play a crucial role in modulating immune functions in cancer patients. Therefore, breast cancer patients were assessed in a psychometric scoring on the patient's religious inclination along with norepinephrine levels, circulating estrogen levels, plasma and PBMC antioxidant activity, con A-induced lymphocyte proliferation, and cytokine production in order to assess the effect of spirituality in the progression of the disease and immunological function.