CHAPTER 12

DISCUSSION

The bi-directional interactions within the neuroendocrine-immune network are complex and vital to the maintenance of cellular and systemic homeostasis and in the prevention of disease development. However, the roles played by the active mediators of the neuroendocrine-immune network have not been fully explored. Although there are several key regulatory molecules in the neuroendocrine-immune network, we have chosen norepinephrine as the neural mediator, estrogen as the endocrine counterpart on resting and activated lymphocytes as the immune aspect of the network. These key mediators have been chosen keeping in mind that dysregulation of the neuroendocrine-immune network due to age-related decline in sympathetic noradrenergic innervation of lymphoid organs precedes loss of cell-mediated immune functions and development of age-associated diseases such as cancer. With this in mind studies were conducted on the three specific objectives identified: The objectives and a discussion of the salient findings are detailed below:
Specific Objective 1A

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

The current study was conducted with the aim of examining the role of estrogen on splenocytes through specific estrogen receptor subtypes ERα- and ERβ- to understand estrogen-mediated immunomodulation through these receptors on proliferation, cytokine production, compensatory cellular mechanisms and intracellular signaling pathways. Results obtained from this study confirm and provide additional findings that the immunomodulatory role of estrogen on splenic lymphocytes is dependent on concentration and its receptor subtypes.

Data from previously published literature shows that the diverse immunomodulatory effects of estrogen on Th immunity (Th1 vs. Th2) vary depending on the type of immune cells or animals used as discussed below. Administration of a low dose of estrogen in vivo to normal mice and mice with autoimmune diseases showed increased Th2 and Th1 cytokine production associated with enhanced CD4 T cell responses [69, 24, 23]. Conforming to these studies, there was a significant increase in T-cell proliferation and IFN-γ production in cells treated in vitro with lower concentrations of 17β-estradiol in the absence and presence of Con A.

In addition, this study establishes the dose-dependent effects of estrogen on proliferation, cytokine production, molecular marker expression, nitric oxide production and antioxidant enzyme activities. The effects of estrogen on splenocyte proliferation, p-ERK/Total ERK expression, p-CREB/Total CREB expression, p-Akt/Total Akt expression, CAT, GPx activities and nitric oxide production are both concentration-dependent and the responses were biphasic in unstimulated cells. In Con A-stimulated cells a biphasic concentration-dependent response was observed in lymphocyte proliferation, IFN-γ production, p-Akt/Total Akt expression, CAT activity and nitric oxide production in response to estrogen treatment. Similar results have been observed by co-incubation of splenic lymphocytes from Wistar strain of rats with lower concentrations of 17β-estradiol in the absence and presence of Con A enhanced T cell proliferation and cytokine production further confirming the concentration-dependent effects of estrogen on Th1 cytokines especially IFN-γ (unpublished data).
Estrogen-induced increase in IFN-γ production as shown in this study has also been reported in splenic lymphocytes and invariant natural killer cells and it may be due to its capacity to modulate the cytokine production at the transcription level [70, 71]. However, contrasting evidence exist for the estrogen’s role to increase IFN-γ either by an increase in its transcription or direct interaction with estrogen response element (ERE) in lymphocytes from patients of autoimmune diseases and rodents [71-73]. In the present study, we clearly demonstrate that in vitro incubation of splenic lymphocytes from Sprague-Dawley rats with 17β-estradiol without and with T cell mitogen, Con A, had no effect on IL-2 production although IL-2 is an important driver of T-cell proliferation. The lack of any effect on IL-2 production by estrogen is in agreement with the finding that prolonged co-incubation of lymphocytes with estrogen not only suppressed IL-2 production but also down-regulated IL-2 receptor expression mediated through the inhibition of IL-2 promoter transcription factors: NF-κB and AP-1 [25]. Another salient feature of this study is that the immunomodulatory effects of estrogen were more pronounced at lower concentrations while higher doses did not alter the Th 1 cell immune responses in unstimulated (proliferation, IFN-γ production, p-ERK, p-CREB and p-Akt expression, CAT activity and nitric oxide production) and Con A-stimulated (proliferation, IFN-γ production, p-Akt expression, CAT, GPx activities and nitric oxide production) splenocytes. Although the production of Th2 cytokine was not measured in this study, it is possible that higher doses of estrogen may have promoted the production of anti-inflammatory cytokine such as IL-10 [12, 74] to inhibit the production of Th1 cytokine, IFN-γ and thereby, modulated Th1 cell immune responses as reported in the present study [69].

Genomic and non-genomic effects of estrogen are transduced by intracellular and membrane ER. ERα and ERβ are differentially expressed on T and B cells with predominant expression of ERα on T cells while B cells have increased presence of ERβ [26]. Incubation of splenic lymphocytes with lower concentrations of ERα agonist, PPT, enhanced T cell proliferation and IFN-γ production but decreased IL-2 production and the effects on proliferation and IFN-γ production alone were reversed by tamoxifen. Similar to estrogen, the high dose of PPT (10⁻⁶ M) did not significantly alter splenocyte proliferation, IFN-γ production, p-ERK expression and nitric oxide production. In
contrast to ERα agonist, incubation of splenocytes with ERβ agonist, DPN, not only enhanced T cell proliferation and IFN-γ production but also increased IL-2 production. The differential modulation of IL-2 by ERα- and ERβ- activation may explain the lack of estrogen-induced effects on IL-2 production.

Further, DPN-treatment of splenocytes altered proliferation, IFN-γ production, SOD, CAT, GPx activities in a concentration-dependent, biphasic manner which was reversed by the antagonist. The inhibition of the T cell immune reactivity by the non-specific ER antagonist tamoxifen suggests that the immune responses are ER-mediated. It is probable that estrogen-induced effects on T cell immunity may be dependent upon the type of immune cells and mediated through ERβ with equal contribution from ERα and membrane receptors [75]. ERα agonists have been shown to reduce the levels of IL-6 and TNF-α cytokines by splenocytes suggesting that estrogen may be exerting immunoprotective effects through ERα to suppress inflammation in trauma-hemorrhage [76]. In a similar animal model of trauma-hemorrhage, estrogen, PPT, and DPN reversed the suppression of T cell proliferation and both Th1 and Th2 cytokine production by Peyer’s patch T cells suggesting that estrogen may act via both ERα/β to confer protection against bacterial infection and sepsis [86]. However, the possibility of these immune responses being mediated through the membrane-bound G protein-coupled estrogen receptor (GPER) needs to be explored as this class of receptors also regulates T cell functions [83].

Estrogen in the absence and presence of Con A; PPT, and DPN enhanced phosphorylation of ERK1/2, CREB, and Akt while tamoxifen was efficient in reversing these effects. An increase in the estrogen- and ERα/β agonist-induced phosphorylation of ERK 1/2, CREB, and Akt in the splenocytes indicates that these intracellular molecular factors are responsible for the immunostimulatory effects on T cell responses. Numerous studies have established a role for ERK/MAPK, CREB, and Akt pathways in estrogen-induced modulation of tumor and neuronal cellular functions [87-90]. However as reviewed in Lannigan et al, 2003, effects of 17β-estradiol on MAPKs are substantially lesser when compared with other growth factor mediated signals in splenocytes also [91]. Similar to these cells, estrogen and both ERα/β agonists augmented cytokine production and MAPK activation in splenocytes in rodents with
trauma-haemorrhage indicating that estrogen’s effects are mediated through ERα/β involving ERK pathway [86, 92].

Estrogen’s activation of CREB in the present study may have been due to the hormone’s effects on cAMP production subsequent activation of p38 MAPK pathway that may be regulated by transcriptional repressor, cyclic AMP response element modulatorα, via ER-dependent mechanism [93, 94]. An increase in the phosphorylation of Ser/Thr kinase, Akt, by estrogen and ERα/β agonists demonstrate the involvement of phosphatidylinositol-3 kinase in the activation of the enzyme, inducible nitric oxide synthase, to modulate the immune functions [95]. Production of NO was enhanced by lower doses of estrogen co-incubated with Con A and ERα/β agonists while it was inhibited by higher doses of estrogen with Con A and ERα agonist suggesting that these effects are concentration-dependent mediated through increased IFN-γ production [96].

Antioxidant enzymes play a crucial role in preventing the deleterious effects of free radicals to damage the cellular structure and functions in the body. Estrogen is known to exert neuroprotective effects on the nervous system in cerebral ischemia and neurodegenerative disorders that are mediated through suppression of cellular oxidative stress by antioxidant enzymes and enhancement of growth factors, brain-derived neurotrophic factor and nerve growth factor [97, 98]. Estrogen treatment of splenocytes enhanced SOD, CAT, and GPx-1 enzyme activities while co-incubation of lymphocytes with ERα/β agonists differentially enhanced these enzymes that are similar to the effects observed in the central nervous system [99, 100]. Co-incubation of splenic lymphocytes with estrogen and T cell mitogen, Con A, markedly increased the T cell proliferation, IFN-γ production, phosphorylation of CREB, activities of antioxidant enzymes, and nitric oxide production indicating the role of immune molecules such as cytokines in indirectly regulating these factors [101-103].

Inhibitors of ERK, and PKA and PKC pathways abrogated the increase in lymphoproliferation and IFN-γ production suggesting that these immune responses by estrogen are mediated specifically through these pathways. Whether blocking MAPK signaling pathway with PD98059 increases MHC class I expression on immune cells thereby altering the immune status remains to be examined but similar effect was found on breast cancer cells (102). The lack of estrogen-induced effects on IL-2 production and
absence of the effects of inhibitors of MAPK, PKA, and PKC pathways suggest that this may be due to inhibition of IL-2 production along with down-regulation of IL-2 receptor expression mediated through the inhibition of IL-2 promoter transcription factors: NF-κB and AP-1 because of prolonged co-incubation of lymphocytes with estrogen (104). Another possible explanation for the absence of IL-2 production even though there was an estrogen-induced increase in p-Akt/Total Akt ratio may be due to a possible suppression of NF-κB by estrogen because IL-2 promoter is regulated by p-Akt expression through nuclear factor κB (NF-κB) sites within these promoters (105, 106). Further studies are critical to the understanding of cross-regulating roles of intracellular signaling cascade molecules in cytokine production.

In summary, our findings suggest that treatment of splenocytes with lower concentrations of estrogen upregulates T cell immune responses that are mediated through specific ER via intracellular signaling mechanisms involving ERK 1/2, CREB, and Akt. An increase in nitric oxide production and antioxidant enzyme activity in a concentration-dependent manner may be responsible for the immunomodulatory and protective effects of estrogen.

**Specific Objective 1B**

**Establish the Influence of α1 and α2-adrenergic receptor (AR) agents (agonists and antagonists) on modulation of splenic lymphocyte functions.**

Cell-mediated immune responses are altered through the modulation of α1- and α2-AR on the splenocytes while estrogen differentially alters the immune reactivity through the α-AR. In the present study, phenylephrine, α1-AR agonist, did not alter lymphoproliferation and suppressed IFN-γ production while clonidine, α2-AR agonist, reduced proliferation of lymphocytes and had no effect on IFN-γ production. A salient finding of this study is a dose-independent increase in IL-2 production by the α1-AR agonist, phenylephrine, that was reversed by its antagonist prazosin and the effect may have been due to receptor density expression on splenocytes. Perhaps, these T cell immune responses are mediated through the presence of α1- and α2-AR on resting T lymphocytes but these immune responses may have been more distinct in the presence of T cell mitogen such as concanavalin A and phytohemagglutinin [33, 107]. Treatment of
lymphocytes with T cell mitogen enhanced the expression of \( \alpha_1 \)-AR on the T lymphocytes that may be responsible for the altered cytokine production such as reduction in IFN-\( \gamma \) and enhanced IL-2 production [33]. The inhibition of proliferation of lymphocytes and lack of cytokine production by \( \alpha_2 \)-AR agonist, clonidine, is in agreement with earlier studies that have reported the inhibition of T cell functions following catecholamine treatment due to decline in lymphocytes by apoptotic mechanisms [35, 108]. While clonidine treatment did not affect IL-2 production by splenocytes, co-incubation of splenocytes with its antagonist, idazoxan, induced a marked decline in IL-2 production suggesting that its actions may have been mediated through the inhibition of imidazoline-2 receptor [109]. A similar inhibition of lymphoproliferation by \( \alpha_1 \)-AR agonist as shown in the present study has been reported earlier in which cellular proliferation of lymphocytes from lymph nodes were inhibited by phenylephrine and reversed by its antagonist, phenotolamine, suggesting that such neural regulation of immunity may be involved in antigen processing and presentation [109]. Both phenylephrine and clonidine enhanced nitric oxide production but their respective antagonists, prazosin and idazoxan, alone also increased its production indicating that these drug-induced non-specific effects may play a role in contraction of the spleen and trafficking of lymphocytes. Clonidine has been shown to enhance nitric oxide production in rat aortal endothelial cells through activation of PI3K/Akt pathway similar to results obtained in our study [110]. These results suggest that a balance in the functioning of immune system is achieved through the immunomodulatory effects mediated through the \( \alpha \)-adrenergic receptors on the splenocytes.

The intracellular mechanisms in \( \alpha \)-AR-induced immunomodulation may be through enhancement and suppression of p-ERK and p-CREB expression by \( \alpha_1 \)-AR agonist, phenylephrine, and \( \alpha_2 \)-AR agonist, clonidine, respectively, while clonidine alone increased p-Akt expression in the splenocytes. These observations in lymphocytes are similar to the increase in phosphorylated ERK and CREB levels observed following phenylephrine treatment of peripheral blood mononuclear cells and cardiac myocytes [107, 111]. It is probable that similar to cardiac myocytes, phenylephrine-induced phosphorylation of CREB in splenocytes may involve several signalling pathways such as p38-MAPK, mitogen- and stress-activated protein kinase-1, and protein kinase A or
through nuclear α1-adrenergic receptors [111, 112]. In general, α2-ARs mediate their action through the activation of inhibitory G protein and inhibition of adenylate cyclase resulting in reduction in cAMP production that is involved in downstream synthesis of CREB. The suppression of p-CREB and also, p-ERK in the lymphocytes in the present study is in agreement with the expected actions of clonidine via α2-ARs. Alternatively, some of the immunosuppressive effects of clonidine may be also effected through the phospholipase-protein kinase C intracellular pathway and by enhancement of p-Akt expression involving phosphoinositide-3-kinase pathway [113, 114]. In the present study, treatment with idazoxan, α2-AR antagonist, reversed the inhibition of p-ERK and p-CREB expression, and the increase in p-Akt expression indicating the specific intracellular actions of clonidine-induced effects on splenocytes. Further, low dose of α2-AR agonist clonidine also significantly enhanced p-NF-κB expression indicating that clonidine-mediated increase in iNOS activity may be through NF-kB cascade [115].

Splenocytes were simultaneously treated with estrogen and α-AR agonists and antagonists to examine the influence of estrogen on proliferation of lymphocytes and cytokine production to understand its role in immunomodulation. Estrogen has diverse effects on Th immunity with either an increase in Th1 or Th2 cytokine production associated with CD4+ T cell responses or augment humoral immunity characterized by increased immunoglobulin production due to IL-10 [12, 23, 24, 69, 74]. Co-incubation of splenocytes with estrogen and α1-AR agonist, phenylephrine, increased IFN-γ and IL-2 production that is primarily due to estrogen-induced response especially, its effect on IFN-γ production [70, 71]. In vitro treatment of splenocytes with estrogen is known to significantly enhance splenocyte proliferation and IFN-γ production in a dose-dependent manner [103]. Further, estrogen alone is known to decrease IL-2 production and down-regulate IL-2 receptor expression mediated through the inhibition of IL-2 promoter transcription factors but co-incubation with α1- and α2-AR agonists increased IL-2 production that was reversed by their antagonists indicating the distinct role of α1- and α2-AR in inducing IL-2 production [25]. Estrogen-induced increase in p-ERK expression and inhibition of p-Akt expression by α1-AR agonist and suppression of p-ERK and p-CREB expression with an increase in p-Akt expression by α2-AR agonist indicates that
the interactive effects of estrogen and α-AR because estrogen is known to increase p-ERK, p-CREB, and p-Akt through specific subtypes of estrogen receptors [103, 92, 94]. Idazoxan, α2-AR antagonist, reversed clonidine-induced increase in nitric oxide production and p-ERK and p-Akt expression in the presence of estrogen suggesting the specific effects of estrogen and α2-AR on the intracellular targets. These data provide evidence for the specific roles for α1- and α2-AR in immune responses and also, the role for estrogen modulating their effects that may have profound effects on alterations in immunity during estrous cycles and reproductive aging.

In conclusion, binding of α1- and α2-AR on splenocytes inhibited proliferation of lymphocytes and IFN-γ production while α1-AR stimulation with phenylephrine enhanced IL-2 production accompanied by increased expression of p-ERK and p-CREB and α2-AR binding increased p-NF-κB and p-Akt expression. Estrogen and α1- and α2-AR antagonists had distinct effects on immune responses and intracellular targets suggesting specific receptor-mediated effects. Further studies are essential to understand the interaction at their receptor level and downstream intracellular signalling pathways to devise therapeutic strategies against female-specific diseases such as autoimmune diseases, hormone-sensitive cancer, osteoporosis, and cardiovascular disorders.

**Specific Objective 1C**

**Establish the Influence of β-Adrenergic Receptor (AR) agents (agonists and antagonists) on modulation of splenic lymphocyte functions.**

Sympathetic NA innervation of lymphoid organs, the release of NE by these nerve fibers, and subsequent binding of NE predominantly to β2-AR on the lymphocytes have been reported to be responsible for the alterations in innate, humoral, and cell-mediated immunity in health and diseases [1, 27-29]. Results from our study have shown that the effects of β1-AR activation on splenocyte functions are not predominant when compared with β2-AR stimulation. β1-AR activation was predominantly immunosuppressive: decreasing splenocyte proliferation and not altering cytokine production, nitric oxide production although it enhanced p-ERK/Total ERK expression and p-Akt/Total Akt expression. However, when in vitro incubation of splenocytes with terbutaline without the
activation of T lymphocytes was examined in the present study, lower doses of terbutaline alone inhibited lymphoproliferation while only higher dose enhanced cytokine production. Although the immunosuppressive effects of β2-AR mediated through Th1 cytokine (IL-2 and IFN-γ) production is well established [40, 42], lower dose of terbutaline (10^{-9}M) treatment of splenocytes in the present study demonstrated a trend for a decline in IL-2 production. An increase in the production of cytokines by 10^{-3}M terbutaline in the absence of activation of T lymphocytes by mitogens in the present study may be due to differential expression of β2-AR on Th1-type lymphocytes or the type of naïve versus effector CD4 T lymphocytes. In agreement with this notion, β2-AR expression on anti-CD3 mAb-activated CD4+ effector Th cells differed from the level on resting cells and higher concentration of NE (10^{-5}M) increased IFN-γ production as a result of Th1 differentiation [116,117]. Similar effects on immune responses following high dose of terbutaline treatment of lymphocytes were obtained in studies involving Wistar strain of rats (unpublished data). In addition, high dose of terbutaline-induced effects on cytokine production was reversed by β2-AR antagonist, propranolol, suggesting that 10^{-3}M terbutaline exerts physiological functions on resting lymphocytes through yet to be determined mechanisms.

Generation of free radicals during immune response and upon neurotransmitter release in the secondary lymphoid organs is known to impair the maintenance of sympathetic NA neurons leading to deficits in the neural-immune interactions during aging and diseases [118]. Antioxidant enzymes are important in the clearance of free radicals and thus, may improve the integrity of sympathetic neuronal activity in the lymphoid organs. Some of the beneficial effects of estrogen in cerebral ischemia and neurodegenerative disorders are believed be due to its ability to suppress cellular oxidative stress by augmenting antioxidant enzyme activities besides increasing the biosynthesis of growth factors [97, 98]. In this study, estrogen enhanced SOD and GPx activity with no effect on catalase activity that was similar to the effects on these enzymes [103]. These effects may be dependent upon the ER subtypes because co-incubation of lymphocytes with ERα/β agonists differentially enhanced these enzymes that are similar to the effects observed in the central nervous system [99, 100]. In contrast to an increase in the activities of SOD and catalase by terbutaline alone, there was a suppression in the
activities of SOD, catalase, and GPX when estrogen was co-incubated with terbutaline suggesting that an increase in the levels of estrogen and NE on the day of proestrous may impair the activities of these antioxidant enzymes leading to loss of sympathetic NA neurons with advancing age [103].

Inducible nitric oxide synthase enzyme is up regulated during inflammation resulting in the production of NO that is known to exert antimicrobial properties such as inhibition of growth of bacteria, viruses, and protozoa besides being a key signaling molecule altering the trafficking of lymphocytes. In this study, terbutaline alone enhanced nitric oxide production and inhibited proliferation of lymphocytes along with an increase in cytokine production while the inhibitor of iNOS reversed the effects of terbutaline suggesting that NO has the ability to modulate T cell immunity. iNOS expression is specifically regulated at the level of gene expression by a variety of transcription factors including NF-κB, STAT-1α, and interferon regulatory factor-1 by IFN-γ through cAMP signaling pathway [119]. Co-incubation with estrogen and terbutaline suppressed nitric oxide production that may be due to the involvement of specific cytokines such as IFN-γ that may regulate its production. In agreement with this finding, we have reported that estrogen alone inhibits nitric oxide production whereas there is an increase in nitric oxide production in the presence of Con A [103].

In summary, we report that terbutaline alone inhibits T cell proliferation and enhanced cytokine production in vitro that is dose-dependent and mediated through ERK, PKA, and PKC pathways, antioxidant enzymes, and NO production. In contrast, estrogen in the presence of terbutaline reversed the terbutaline-induced effects on immune responses and molecular targets suggesting that there is differential modulation of β-AR in the presence of estrogen that may be responsible for reproductive aging and the development of female-specific diseases such as hormone-sensitive cancers, autoimmunity, osteoporosis, etc.

Specific Objective 2A

Involvement of Cellular Mechanisms in α1 and α2-AR-induced effects on ER (+) and ER (-) human breast cancer cell lines MCF-7 and MDA MB-231.
α₁-AR and α₂-AR agonists differentially modulate the proliferation, expression of pro-angiogenic factors and activation of signaling molecules in ER (+) MCF-7 and ER (-) MDA MB-231 cells in vitro. The present study has shown that α₁-AR agonist phenylephrine selectively enhances the proliferation of ER (+) breast cancer cell line MCF-7 alone by enhancing the expression of VEGF A, NO and activating p-ERK, p-CREB and p-Akt signaling pathways. Although the direct effects of α₁-AR stimulation on tumor cell proliferation and expression of pro-angiogenic factors have not been extensively studied α₁b expression was correlative with increased proliferation, decreased apoptosis, poor cancer-specific survival and increased tumor recurrence [120]. In our study, the proliferation of ER (-) MDA MB-231 cells was not altered by α₁-AR stimulation indicating that the relative distribution of α₁-AR subtypes may differ on these cell lines or part of the signaling mechanism may involve the estrogen receptor.

The expression of pro-angiogenic factors such as VEGF A and NO were enhanced in ER (+) MCF-7 cell line and decreased in ER (-) MDA MB-231 cell line treated with phenylephrine. Binding of VEGF A to its high affinity receptor VEGF-R2 has been shown to enhance nitric oxide production in microvascular endothelial cells through the PI3K/p-Akt pathway thereby enhancing cellular survival by inhibiting pro-apoptotic pathways [121-125]. Also, VEGF A binding to VEGF-R2 in endothelial cells has been shown to trigger PKC-mediated phosphorylation of sphingosine, leading to activation of H-Ras, c-Raf-1 and ERK1/2 and induce phosphorylation of CREB in human umbilical vein endothelial cells (HUVEC) [126-128]. This is in agreement with our study where phenylephrine treatment with ER (+) MCF-7 cells enhanced VEGF A and nitric oxide production through enhanced p-Akt, p-ERK and p-CREB expression. VEGF C induced by phenylephrine in ER (-) MDA MB-231 cells can bind to VEGF-R3 receptors and activate the adapter proteins Shc, Grb2-Sos and mediate ER1/2 phosphorylation in a Ras-dependent and Ras-independent mechanism in lymphatic endothelial cells [129]. Also, MDA MB-231 cells transfected with exon-deleted version of estrogen receptor (ER)-α enhanced the expression of VEGF in MDA MB-231 cells, thereby indicating the role of ER in activating VEGF signaling in breast cancer cells [130].

On the other hand, our data indicate that α₂-AR agonist clonidine similarly enhances the proliferation of ER (+) MCF-7 and ER (-) MDA MB-231 cells in vitro. The
pro-proliferative effects of \( \alpha_2 \)- AR agonist clonidine on DMBA-induced mammary tumors in rats, mouse mammary tumors and human breast cancer cell lines like MCF-7, have been previously studied [131-133]. Also, clonidine has been shown to inhibit IL-1\( \beta \) induced VEGF expression in human retinal pigment epithelial cells by suppression of p38MAPK and MEK1/2 signaling cascades [134]. Corroborative with these findings, activation of \( \alpha_2 \)- AR signaling in MCF-7 cells did not alter VEGF A expression, decreased VEGF C and nitric oxide production down-regulated p-CREB expression and did not alter p-ERK and p-Akt expression. Similar findings were observed in ER (-) MDA MB-231 cells where no significant change was observed in VEGF A, VEGF C and p-ERK expression and decreased NO, p-CREB and p-Akt expression pathways and did not alter p-ERK expression. Decreased p-CREB expression by clonidine may be due to the distinctive effects of \( \alpha_2 \)-adrenoceptor stimulation on cAMP levels [133].

\( \alpha_1 \)- AR blockade using prazosin significantly decreased the proliferation and VEGF A production by ER (+) MCF-7 cells, although it did not alter proliferation and VEGF expression in ER (-) MDA MB-231 cells. In agreement with these findings, treatment of MCF-7 cells with prazosin reversed epinephrine-mediated increase in proliferation [133]. Also, \( \alpha_2 \)- AR antagonist idazoxan, significantly decreased the proliferation of MCF-7 and MDA MB-231 cells and VEGF C production by MCF-7 cells. Treatment of experimentally induced mammary tumors in animals using \( \alpha_2 \)- AR blocker rauwolscine significantly diminished tumor growth in vivo and in vitro [131, 135]. Also, treatment with \( \alpha_2 \)- AR blocker yohimbine decreased proliferation of MCF-7 cells in vitro by reversing agonist-mediated effects on cAMP [44]. In PC-2 and PC-3 pancreatic cancer cell lines, yohimbine treatment inhibited proliferation by inducing apoptosis [136].

In conclusion, binding of \( \alpha_1 \)- AR on cancer cell lines differentially modulated the proliferation and expression of pro-angiogenic factors in vitro depending upon the presence or absence of estrogen receptors. \( \alpha_2 \)- AR agonist on the other hand similarly enhanced proliferation and inhibited expression of pro-angiogenic factors in ER (+) and ER (-) breast cancer cell lines. \( \alpha_1 \)- and \( \alpha_2 \)-AR antagonists had distinct effects on cancer cell proliferation, expression of pro-angiogenic factors and intracellular targets suggesting specific receptor-mediated effects. These results imply a differential role for sympathetic
interactions in influencing outcomes in hormone-responsive and non-responsive breast cancers from which parallels can be drawn to develop treatment strategies by targeting sympathetic modulation of tumor survival through angiogenic factors. Further studies are required to explore the use of adrenergic receptor agents either alone or as part of combination therapy in the prevention and treatment of breast cancer in women.

Specific Objective 2B

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

Although several studies have demonstrated the presence and β-AR-mediated modulation of growth and metastasis of breast cancer cells, differential characterisation of these signals on the basis of their hormone responsiveness has not been established. In this study, evidence obtained from adrenergic stimulation of ER(+) and ER(-) breast cancer cell lines have shown contrasting effects on proliferation, production of angiogenic molecules like VEGF C, release of soluble mediators such as NO and expression of molecular markers like p-ERK, p-CREB and p-Akt. Proliferation of ER (+) cell line MCF-7 was enhanced in a β-adrenergic receptor-mediated manner upon stimulation with terbutaline and reversed upon blockade with propranolol. Similar increase in MCF-7 cell proliferation and tumor growth in ovarian cancers has been reported in response to terbutaline which may be attributed to activation of β2-AR-mediated survival signalling pathways through PKA [137]. Lesser density of β2-AR on MCF-7 cells may help these cells to down-regulate terbutaline-mediated increase in cAMP, and favour its effects on PKA-mediated activation of survival signals, contributing to enhanced proliferation [138].

Concomitant with the increase in proliferation, a significant increase in nitric oxide production was observed through β-AR mechanism which was reversed by beta blockade using propranolol. The increase in NO may be due to the expression of endogenous nitric oxide synthase which is implicated to promote the growth of breast, cervical, central nervous system, laryngeal and head and neck cancers [139-143] by regulation of angiogenic mechanisms through VEGF-NO-cGMP pathway [144-146]. Our study shows that stimulation of β-AR significantly increased the production of VEGF C which was
reversed upon co-incubation with propranolol in ER (+) cells although the increase was subtle and only in the high dose probably due to the lesser distribution of β2-AR on MCF7 cells [138].

Contrary to the effects observed in MCF-7 cells, β-AR stimulation of ER (-) cell line MDA MB-231 resulted in decreased proliferation which was not reversed upon co-incubation with selective β-antagonist propranolol. This may be due to increased terbutaline-mediated cAMP production in the higher doses (10^{-6} M and 10^{-9} M) coupled with the enhanced β-AR expressing phenotype of these cells leading to cAMP-mediated decrease in cell proliferation [138]. Nitric oxide production was however enhanced by activation of β-AR-mediated signals and ameliorated upon β-blockade using propranolol in ER(-)cell line MDA MB-231 similar to ER(+) MCF-7 cells possibly through endogenous activation of nitric oxide synthase [139-143]. VEGF C production however, was differentially regulated by β-AR stimulation in ER (-) cell line MDA MB-231 when compared with the ER (+) cell line MCF-7. While terbutaline treatment increased VEGF C production in MCF-7 cells, it decreased VEGF C production in MDA MB-231 cells possibly due to the prolonged cAMP response in these cells [138]. This may indicate a role for ER in modulating β-adrenergic modulation of cancer progression and pathogenesis.

Stimulation of β-ARs by terbutaline is characterised by enhanced phosphorylation of p-ERK, p-CREB and p-Akt in the ER (+) cell line MCF-7. While β-blockade using propranolol significantly reversed the phosphorylation of CREB and Akt in the ER (+) MCF-7 cells, the production of p-ERK was further enhanced which may be due to the reverse agonistic effects of propranolol on the beta adrenergic system. Use of inhibitors to PKA and PKC, ERK and Akt pathways significantly reversed the terbutaline-mediated increase in MCF-7 cell proliferation indicative of the role of these three pathways in beta AR-mediated ER (+) cancer survival signalling. Stress-induced increase in catecholamines have been shown to enhance proliferation and growth of ovarian cancers in mice through β-adrenergic receptor-mediated increase in PKA, ERK and CREB signalling [137]. Similar to our study, treatment with β-blocker propranolol significantly reversed the norepinephrine-induced increase in the invasiveness of ovarian [147] and pancreatic cancers by inhibition of cAMP/PKA and CREB signals [148-149]. Also
treatment with terbutaline significantly enhanced the production of p-Akt by MCF-7 cells that is known to contribute to increased tumor aggressiveness, development of hormone resistance and increased metastasis possibly through GPCR-mediated transactivation of receptor tyrosine kinases or EGFRs [150-152].

On the contrary, in ER (-) MDA MB-231 cells, p-ERK was not enhanced by terbutaline stimulation of β-ARs rather only p-CREB and p-Akt signals were amplified. Synergistic effects of ER-β and β-AR have been shown to activate phosphorylation of ERK1/2 in lung adenocarcinomas. However, lack of estrogen receptors may explain the absence of ERK activation in MDA MB-231 cells. Low doses of terbutaline significantly decreased p-ERK expression while higher doses did not alter it indicating that terbutaline-mediated survival signals in ER (-) MDA MB-231 cells are not transduced through p-ERK. PKA-mediated increase in CREB have been shown to enhance invasiveness and metastatic ability of ovarian and pancreatic cancers through classical β2-AR signalling pathway [147-149]. Beta blockade using propranolol however, significantly decreased terbutaline-mediated increase in p-CREB and p-Akt production indicative of a role for these signals in mediating survival signalling in ER (-) cells. Corroborative evidence of this was obtained using pathway inhibitors for PKA and PKC, ERK and Akt, which showed that ERK blockade did not affect terbutaline-mediated survival signalling in ER(-) cells.

β1-AR agonist dobutamine did not affect proliferation, VEGF A and VEGF C production but increased NO production of ER (+) MCF-7 cells although it increased the proliferation and decreased VEGF A and VEGF C production by ER (-) MDA MB-231 cells. p-ERK and p-CREB expression by ER (+) MCF 7 cells and ER (-) MDA MB-231 cells was decreased while p-AKT expression was increased.

Specific Objective 3

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

Chronic illness stress-associated alterations in immunological, neurochemical and endocrinological functions in ovarian and other cancers through higher levels of tissue
catecholamines, greater tumor burden and more invasive growth of ovarian carcinoma cells are mediated through activation of β2-AR signaling [137,153]. PBMCs obtained from breast cancer patients have provided evidence of the neuroendocrine-immunomodulatory effects of spirituality which has been shown to reduce stress and cortisol-mediated immunosuppression in patients with chronic illnesses through activation of neuroendocrine-immune mechanisms [154-158].

In our study also, we have obtained correlative evidence of increased Con A-induced T-cell proliferation in spiritual women with breast cancer compared with their non-spiritual counterparts. Further we have also shown that these effects are mediated through specific molecular signalling pathways such as mediated through the PKA and PKC pathways by using signal inhibitory molecules. Also, these effects were not observed in LPS-mediated B-cell proliferation indicative that the beneficial effects of spirituality on the neuroendocrine-immune network may be limited to cell-mediated immune responses. Since sympathetic noradrenergic fibres in lymphoid organs are found in close apposition to T-cell rich regions and steer clear or sparsely innervate B-cell rich pockets [27], it is possible that sympathetic stimulation may have an important role in mediating the beneficial effects of spirituality in chronic-illness-associated immunosuppression.

Data obtained has shown that there was a significant decline in the Con A-induced proliferative capacity of PBMCs isolated from nonspiritual women with breast cancer compared to their spiritual counterparts suggesting that spirituality enhances con A-induced proliferation of PBMCs in cancer patients. Although Con A-induced TNF-α and IL-6 production was not altered with age, disease or spiritual inclination, there was a significant increase in Con A-induced IL-2 and IFN-γ production by PBMCs isolated from spiritual women with breast cancer compared with non-spiritual women with breast cancer. This is in agreement with findings from similar studies where women who rated spiritual expression as more important had greater numbers of circulating white blood cells and total lymphocyte counts and spiritual inclination was directly correlative with increased helper and cytotoxic T-cell counts [157]. Also, there was a significant increase in the Con A-induced nitric oxide production in spiritual women compared to non-spiritual women.
Spirituality was found to modulate compensatory mechanisms such as antioxidant enzyme activities that are known to decline with aging, thereby resulting in enhanced free radical load which leads to reduced cell-mediatd immune responses facilitating the development of age-related diseases like autoimmunity, infectious diseases and cancer [1,15]. In resting lymphocytes, a breast cancer-related decline was observed in SOD activity, while CAT and GPx-1 activities declined with age and breast cancer and spiritual young women had elevated CAT activity compared with nonspiritual young women. The extent of lipid peroxidation in resting PBMCs was significantly enhanced in spiritual and non-spiritual women with breast cancer compared with young and middle-aged controls. In LPS-stimulated cells however, SOD activity was higher in middle-aged controls compared with young and declined in women with breast cancer while CAT and GPx activities declined with age and breast cancer. CAT activity was enhanced in spiritual young women alone while GPx-1 activity was enhanced in young, middle-aged and women with breast cancer compared to their respective non-spiritual controls. Although Con A-induced SOD and CAT activities did not alter with spiritual status, there was a significant decrease in PBMC Gpx-1 activities in non-spiritual young and middle-aged women compared to spiritual counterparts.

Correlation analysis using linear regression on scatterplot data showed a significant positive correlation between spirituality and physical (A), emotional (B) and functional well-being (C) as reported by the survey collected from the patients themselves. Studies from other laboratories have shown that greater quality of social support is associated with lower cortisol concentrations in women with metastatic breast cancer, which is indicative of healthier neuroendocrine functioning [156]. It is possible that spirituality extends similar benefits on the neuroendocrine-immune network, by reducing stress and distress possibly by modulating sympathetic cascades as seen in psychotherapy for breast cancer patients [154, 159, 160]. In our study, we have also reported a significant positive correlation was obtained between Spirituality and Con A-induced PBMC proliferation, Con A-induced PBMC nitric oxide production and Con A-induced GPx-1 activity in PBMCs isolated from women with breast cancer. There was a significant increase in the p-ERK and p-CREB expression of Con A-stimulated PBMCs isolated from spiritual women with breast cancer suggesting that
spirituality enhanced Con A-induced p-ERK and p-CREB expression. Pathway inhibitor data suggests that the beneficial effects of spirituality in cancer may be transduced through the PKA/PKC pathway. Thus taken together these results suggest that apart from the beneficial psychosocial effects on physical well-being, emotional well-being and functional well-being of cancer patients, spirituality also enhances cell mediated immune functions as measured by Con A-induced proliferation of PBMCs in cancer patients through activation of PKA pathway, enhanced NO production and GPx-1 activities. However, further studies are warranted in order to ascertain the pathways involved in modulating neuroendocrine-immune mechanisms in health and disease.