LYMPHOCYTE NF-KB AND DEMANDS OF ILLNESS MEASURES FROM
WOMEN WITH STAGE II-IV BREAST CANCER DURING A COMPLEMENTARY
YOGA INTERVENTION

By
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Abstract
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Breast cancer survivors are challenged by many psychosocial issues that increase
the burden of survivorship and overall stress. Increased psychosocial distress alters
nuclear expression of NF-κB, a regulator of gene transcription activities involved in
innate immunity. Interventions that target psychosocial stressors may attenuate
psychosocial distress and benefit immunity. The purpose of this study was to examine
the impact of an 8-week Iyengar yoga intervention on psychosocial stressors and nuclear
expression of NF-κB in rIL-2 and PHA/PMA stimulated lymphocytes of women with
breast cancer. Female subjects, previously diagnosed with stage II-IV HER-2+ breast
cancer and receiving Herceptin®, volunteered to join yoga (n=7) or treatment as usual
(n=3) group. Lymphocytes obtained pre-(T₁) and post-(T₂) intervention were stimulated
with rIL-2 or PHA/PMA. Cytosolic (C) and nuclear (N) extracts were analyzed for NF-
κB by luminescence. Demands of Illness Inventory (DOII) measured hardships or
psychosocial stressors at T₁ and T₂. Data were analyzed by ANOVA with repeated
measures, independent t-tests or Mann Whitney U tests. Because clinical treatment
changes were required for the management of disease, the effects of yoga on lymphocyte

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NF-κB stimulation index was not discernable. NF-κB stimulation index was indirectly correlated with nuclear NF-κB expression in unstimulated lymphocytes (rIL-2 stimulation index, \( R^2 = 0.817, p < 0.001 \); PHA/PMA stimulation index, \( R^2 = 0.455, p = 0.016 \)), independent of group and time. This relationship indicated that greater nuclear expression of NF-κB occurred in those lymphocytes having lower baseline NF-κB nuclear expression. Among all subjects, NF-κB N:C ratio in unstimulated lymphocytes was indirectly associated with hardships associated with breast cancer and its treatment that impact personal meaning (\( T_1: R^2 = 0.4134, p = 0.045 \)). PHA/PMA stimulation index of NF-κB was indirectly associated with overall DOII scores (\( R^2 = 0.4232, p = 0.042 \)), and hardships associated with monitoring treatment and disease related symptoms (\( R^2 = 0.772, p = 0.032 \)). These relationships indicated that those responders reporting increased hardships due to breast cancer disease also had lower stimulation index of lymphocyte NF-κB. The biological relevance of these results requires further investigation; however, these findings lend support to the hypothesis that NF-κB regulation and illness-associated stressors are inter-related. Further explication of the complex interplay of both types of variables could benefit breast cancer survivors.

**Key Words:** yoga, complementary medicine, breast cancer, NF-κB, immune function, psychoneuroimmunology
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CHAPTER ONE

Introduction

Nuclear factor kappa B (NF-κB) proteins are members of the NF-κB/Rel family of transcription factors in lymphocytes, which are involved in activating gene expression responses leading to host defense (12). NF-κB proteins are among a larger group of κB motifs on gene regulatory elements and are capable of inducing transcription of genes (12). NF-κB/Rel proteins are derived from two sources: 1) NF-κB1 (p50) and NF-κB2 (p52), proteolytic products of p105 and p100, respectively and 2) Rel proteins RelA (p65), RelB and c-Rel (12, 25-27). All NF-κB/Rel proteins contain a Rel-homology domain (RHD) or Rel-homology region (RHR), which mediates DNA binding dimerization, nuclear translocation, and binding to inhibitor proteins (IκB) (12, 27). In unstimulated cells, NF-κB proteins are sequestered in the cytosol in an inactive form, due to an association with the cytosolic inhibitory protein, IκB (2, 12, 26). When a cell membrane is stimulated via ligand binding, receptor mediated activation allows NF-κB dimers to dissociate from IκB and translocate to the nucleus, thereby regulating transcriptional activities of genes involved in specific immune responses (2, 5, 12, 26, 35). Altered regulation of NF-κB and its genes in lymphocytes is associated with pathological conditions of the immune system, including B and T cell lymphomas (16, 44). For example, a defect in the NF-κB1 phenotype results in complications with antibody (Ab) production and decreased T cell proliferative responses; whereas a defect in the RelB homology results in the development of T cell mediated inflammatory diseases (12).
The overall role of NF-κB in immune response is mediated through its ability to activate genes encoding for regulators of cell proliferation and apoptosis (25). In addition, NF-κB has been referred to as a transcription factor that regulates lymphocyte function through its production of various chemokines and cytokines, which act to stimulate the migration and maturation of lymphocytes (25, 43).

The immune system is comprised of tissues, cells, and molecules whose primary function is to maintain internal homeostasis by destroying invading infectious organisms. It is also responsible for fighting against the growth, multiplication and the metastasis of tumors through many different mechanisms (52). Natural killer (NK) cells play a major role in innate immunity, tumor surveillance (52, 55) and the prevention of metastases (64). Individuals with cancer often experience dysregulated immune function (32, 55, 63). For example, defects in perforin-dependent natural killer cell activity (NKCA) and phytohemagglutinin (PHA)-stimulated proliferation have been observed in survivors with metastatic melanoma (30). Other T-lymphocyte functions may also be impaired in breast cancer survivors (35, 55). Shevde et al. (54) reported that families with high incidence of cancer, particularly breast cancer, had lower than normal NK and T cell cytolytic functions, which were associated with impaired responses to interferon-alpha and interleukin-12.

Psychological, psychosocial and emotional stressors have been associated with disease progression and vulnerability to illnesses (7). Psychosocial stress may contribute to the etiology and progression of pathologies such as viral infections, impaired wound healing, HIV and cancer (7). Alterations in neuroendocrine-immune pathways can mediate the effects of psychosocial stressors on cancer progression (52). Breast cancer
survivors may demonstrate high levels of chronic stress (52). Under conditions of chronic stress, T cell function may be compromised, indicating a less effective response to defend against viruses, bacteria or tumors (52). Persons with advanced cancer may also have fewer circulating NK cells (52). Under conditions of chronic stress, nuclear NF-κB expression in lymphocytes has also been demonstrated to decrease (43). Therefore, it has been hypothesized that chronic stress related decreases in lymphocyte nuclear NF-κB expression might be linked to decreased secretion of cytokines that could result in negative regulation of immune responses (43).

Psychosocial interventions providing relaxation-techniques training have been reported to enhance NK cell function through counterbalancing the effects of stress on immunity and slowing disease progression. There is an increase in the prevalence of complementary/alternative medicine (CAMs), including relaxation-techniques training, among individuals with life threatening diseases such as HIV or cancer (53). Currently, the most prevalent form of CAMs used among breast cancer survivors includes a combination of tai chi, yoga, or chi gong because of the survivors' belief that these forms of activity reduce stress as well as benefit the immune system (53).

Several different tools have been developed in order to try to measure empirically psychosocial demands associated with different illnesses. The Demands of Illness Inventory (DOII) developed by Haberman et al. (29), is one such tool.

Demands of illness refer to the hardships or stressors that require coping or adjustment response to illness (29). The original DOII included 125 items organized into seven different subscales. These subscales include: 1) physical symptoms, which are the somatic responses of illness and anxiety, 2) personal meaning, which is the priorities,
values and goals that change with the illness, 3) family functioning, including the aspects of decision making, adaptation, and integration in family and work situations, 4) social relationships including changes in social activities as well as helping others deal with the illness, 5) self-image, which is changes in physical appearance or feelings of attractiveness, 6) monitoring symptoms, including vigilance to new bodily sensations, preoccupation with symptoms, fears of recurrence, or the progressive nature of the disease, and 7) treatment issues, which includes accommodation to regimen, treatment evaluation, or the relationship with the providers. The current study used a revised version of the DOII that included 66 items, five subscales, and two items that measured global perception of demands.

Statement of Problem

NF-κB has an important role in immune function, leading to the activation of lymphocytes, including NK cells, which are known to be important in tumor surveillance. Breast cancer survivors may have a compromised immune system and many turn to complementary/alternative medicine (CAMs) as a means to provide immunological benefits. No studies have examined the effects of complementary Iyengar yoga on the nuclear expression of transcription factor NF-κB in lymphocytes. The purposes of this study are: 1) to examine the impact of a complementary Iyengar yoga intervention on nuclear expression of NF-κB in rIL-2 and PHA/PMA stimulated peripheral blood lymphocytes from women with stage II-IV HER2+ breast cancer; 2) to examine the effect of a yoga intervention on psychosocial subsets, as measured by the DOII, in women with
stage II-IV HER2+ breast cancer and 3) to examine associations between nuclear NF-kB expression and psychosocial outcomes as measured by DOII.

**Hypotheses**

The hypotheses of the study were that 1) yoga intervention would increase nuclear expression of NF-κB in stimulated lymphocytes as compared with that from breast cancer survivors who received treatment as usual (TAU); 2) yoga intervention would decrease DOII scores; and 3) lymphocyte nuclear NF-κB expression would be negatively correlated with DOII scores.

**Delimitations**

The results of this study are limited to the effect of an Iyengar yoga intervention in women with stage II - IV HER2+ breast cancer.

**Limitations**

This study is limited by the ability to stimulate increased nuclear NF-κB expression in lymphocytes with rIL-2 and PHA/PMA and by the sensitivity of the ELISA assay to detect NF-κB. The effects of clinical treatments, including chemotherapy and drug interactions on psychosocial and NF-κB measures, also limit this study. It was also limited to compliance of the women with yoga practice. The small sample sizes of the yoga and treatment-as-usual groups limit the generalizability of the findings.
CHAPTER TWO
LITERATURE REVIEW

Members of NF-κB Family

The NF-κB family of transcription factors consists of homo- and heterodimers of five different members of the Rel family (12, 25). These include NF-κB1 (p105/50), NF-κB2 (p100/52), RelA (p65), RelB and c-Rel (12, 25-27). NF-κB1 and NF-κB2 are initially long polypeptide chains that are cleaved after transcription, resulting in DNA binding subunits p50 and p52, respectively (12, 27). The Rel homology domain characterizes members of the NF-κB family and contains a nuclear localization sequence. Rel homology domains function in sequence specific DNA binding, dimerization and interaction with IκB inhibitory proteins (12, 27).

Members of the NF-κB family dimerize to form homo- or heterodimer complexes, which ultimately control transcription. Each of these complexes is involved in different responses to stimuli and has different effects on transcription (12). NF-κB1 and NF-κB2 do not contain the domain required to activate transcription (12, 68). Therefore, these members are thought to act as repressors by preventing binding to the κB site (12, 27, 68). A highly conserved 300 amino acid N terminal Rel homology domain is shared by NF-κB/Rel proteins (26). This domain is responsible for DNA binding, dimerization and association with IκB inhibitory proteins (26). Rel-A, Rel-B and c-Rel contain the domain that can activate transcription (12). Immune functions of NF-κB/Rel include regulation of cell proliferation, functional differentiation of immune cells, migration and maturation
of lymphocytes, growth control proteins, adhesion molecules, major histocompatibility complex (MHC) proteins, co-stimulatory molecules, as well as cytokine production (27). The κB binding site is present in many regulatory regions of genes involved in innate (27).

**Lymphocyte NF-κB Activation Pathway**

Activation of NF-κB in lymphocytes is a rapid process and can be stimulated by proinflammatory cytokines, and is activated under conditions of stress (5). NF-κB has been referred to as a transcription factor that can regulate lymphocyte function (43). Various chemokines and cytokines produced in response to activation of NF-κB act to stimulate the migration and maturation of lymphocytes (25). Therefore, nuclear expression of NF-κB is increased during lymphocyte activation and the secretion of cytokines (43).

Inactive NF-κB proteins and Rel family members remain sequestered in the cytoplasm due to their association with IκB cytosolic inhibitory proteins (2, 12, 26). The association of IκB proteins with NF-κB requires conserved motifs called ankyrin repeats (27). IκB inhibitor proteins are capable of binding to NF-κB dimers with different affinities (12). The IκB family of proteins includes IκBα, IκBβ, IκBε, IκBγ, Bcl-3, and IκBR. Carboxyl-terminal regions of NF-κB precursor proteins NF-κB1 (p105) and NF-κB2 (p100) can also function as inhibitors (2, 12, 26, 27). An additional member of the IκB family that functions solely in the nucleus to inhibit NF-κB is IκBζ (12). NF-κB2 may function both as an IκB and as a heterodimeric partner to RelB, because it can result in the release of NF-κB1/RelB heterodimers (25).
As illustrated in Figure 1, NF-κB activation is initiated by specific ligand receptor binding at the cell surface. Each specific receptor uses a distinct variety of adaptor molecules and signaling enzymes to activate NF-κB (12, 50). Stimuli of NF-κB activation include tumor necrosis factor alpha (TNFα), interleukin-1 (IL-1), IL-2, lipopolysaccharide (LPS), phorbol esters, okdaic acid, serum growth factors and nitric oxide (27). The release of NF-κB from its inhibitor protein IκB involves phosphorylation of IκB by IκB kinases, IKKα and IKKβ, at serine residues and the

**Fig 1 NF-κB Mechanism of Activation.** Receptor-mediated cell signaling pathways leads to phosphorylation (P) of IκB by IκB kinases (IKK), and the subsequent phosphorylation-dependent multi-ubiquitylation (U). This results in degradation of IκB, and the release of NF-κB. NF-κB dimers translocate to the nucleus, bind to specific κB sites located in the promoter region of many genes and regulate transcriptional activity of genes (12).
subsequent phosphorylation-dependent multi-ubiquitinylation at lysine residues (12, 26, 27). This results in degradation of IκB by an ubiquitin-dependent proteasome and the release of cytosolic NF-κB, exposing the nuclear sequence (12, 26, 27). NF-κB dimers translocate to the nucleus, bind to specific κB sites located in the promoter region of many genes and regulate transcriptional activities of gene expression involved in certain immune functions.

For example, NF-κB activation regulates the transcription of cytokines involved in cell proliferation and apoptosis (5, 12, 26). In lymphocytes, NF-κB activation is important for the expression of IL-2R α chain, IL-2, T cell receptor (TCR) β chain, MHC-I, MHC-II, granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor α (TNF-α) and interferon β (15). Prolonged activation of NF-κB can result in increased IκB synthesis leading to an increase of NF-κB being sequestered in the cytoplasm in its inactive form (5).

**Inducers of NF-κB Activation in Lymphocytes**

The inducers phorbol 12-myristate 13-acetate (PMA)/phytohemagglutinin (PHA)/ionomycin and IL-2 are often used in the activation of NF-κB in lymphocytes and were the methods of activation in this study. For these reasons, PHA/PMA/ionomycin and IL-2 methods of activation will be the primary focus of this section. Additionally, the cells primarily being activated in this study are T cells and NK cells, and therefore the cell signaling pathways discussed are primarily focused on these cell subsets.
IL-2 Induction of NF-κB

IL-2 is a 15 kDa glycoprotein that is both produced by T cells and acts on T cells as a growth factor. IL-2 also augments NK cell lytic activity (14). IL-2 stimulation occurs through the IL-2 receptor (IL-2R), and activates pathways that ultimately lead to NF-κB activation. The IL-2R on NK cells and activated T cells has three different subunits (36). The α chain subunit is involved with IL-2 binding, whereas the β and γ chains are involved with transducing cell signals (36). It has been proposed that IL-2 induced activation of NF-κB may occur through various cell signaling pathways (Fig. 2).

Figure 2. Activation Signaling Pathway
For example, following IL-2R ligand binding and phosphorylation, an intracellular signaling complex (Shc-Grb2-Sos trimer) is formed which leads to the activation of Ras (23, 49). Ras activation leads to the tyrosine phosphorylation of Raf, which in turn activates mitogen-activated protein kinase (MEK) (23). A second mechanism involves the Shc-Grb2-Gab trimer and leads the activation of P13K, which in turn activates protein kinase B (PKB)/serine-threonine kinase (Akt) (23, 27, 36). Akt then functions to activate MEK, which leads to the phosphorylation and activation of the extracellular signal-regulated kinase (ERK)-2 and the p38 MAP kinase pathways in certain T cell lines and primary T cells (23, 36). In NK cells, the above pathway will only result in the activation of the ERK2 pathway (36). Activation of MEK, as in the two above mechanisms, can cause IKK activation, leading to degradation of IκB and the subsequent NF-κB activation (31). A third mechanism involves IL-2 activation of Janus Kinase (JAKs), which causes the tyrosine phosphorylation of STAT3 and STAT5 in both NK and T cells (36). STAT3 and STAT5 are both capable of causing the nuclear translocation of transcription factors (23, 36). All of the above described pathways ultimately act to induce the activation of an IκB kinase pathway resulting in the release of NF-κB from its cytosolic inhibitory protein, IκB, and allowing activated NF-κB to translocate to the nucleus (27).

**PMA/Ionomycin/PHA Induction of NF-κB**

Phorbol 12-myristate 13-acetate (PMA), PHA and ionomycin are often utilized in cell stimulation procedures and result in a global stimulation of all lymphocytes in peripheral blood samples. PMA is a phorbol ester that is often used, in conjunction with ionomycin, a calcium ionophore, for the induction of NF-κB activation. PMA and
ionomycin, mimic activation signals following T cell receptor (TCR) and co-stimulatory receptor ligand binding (3, 61). Phorbol esters substitute for unsaturated diacylglycerol requirement for activation of protein kinase C (PKC, PKC-θ in T cells) and directly activate the enzyme (3, 15, 61). Through a mechanism that is not fully understood, PKC results in the activation of IκB kinase (IKKB), leading to the nuclear translocation of NF-κB (3, 10). In lymphocytes where calcium is required for activation of PKC, PMA/ionomycin also mimic IP3 receptor binding that causes Ca$^{2+}$ to be released into the cytoplasm and triggers Ca$^{2+}$-release activated channels (CRAC) on the plasma membrane. This results in the influx of extracellular Ca$^{2+}$ that activates phosphatase calcineurin, which ultimately leads to PCK/IKK activation and the subsequent activation of NF-κB (41).

**NK Cells and Tumors**

The immune system is responsible for fighting against the growth, multiplication and metastasis of tumors through many different mechanisms (52). Many different cell subsets are involved in immune responses. However, NK cells, in particular, are integral to the immune system’s response against tumors. Thus, NK cell function will be the primary focus of this paper.

NK cells play a major role in innate immune surveillance. NK cells conduct non-MHC restricted cytolysis (8), as well as participate in a variety of non-specific innate immune responses to microorganisms (6), virally infected cells (21, 65) and NK-sensitive tumors (6, 65). NK cells also have an important role in the prevention of tumor metastases (63). In vitro, tumor cytolysis by NK cells is conventionally measured by
natural killer cell cytolytic activity (NKCA). Compromised NKCA has been associated with both development and progression of certain cancers (45, 55). NK cell and lymphocyte-activated killer (LAK) cell cytotoxicity can be enhanced by cytokines such as interferon-\(\gamma\) (IFN-\(\gamma\)) and interleukin-2 (IL-2) (63).

NF-\(\kappa\)B is also an important regulator of NKCA, possibly through the regulation of perforin, granzyme A and granzyme B gene expression (67). Perforin is a pore forming protein, which is released from cytotoxic granules of cells, such as T cells and NK cells, and ultimately triggers apoptosis of the target cell (47). Granzyme A and granzyme B are serine proteases, which are localized in the cytoplasmic granules of T cells and NK cells, triggering target cell death. The expression of perforin, granzyme A and granzyme B mRNA is controlled through \textit{cis} regulatory elements in the promoter and enhancer regions of genes (67). Transcription factors, such as NF-\(\kappa\)B, bind to the \textit{cis} regulatory elements, thus regulating the synthesis of mRNA for genes such as perforin, granzyme A and granzyme B (67).

**Breast Cancer and NK Cell Function**

Individuals with cancer often have impaired immune function (32). Breast cancer survivors may have decreased numbers of circulating NK cells, as well as a decreased NKCA (55, 63). Furthermore, this characteristic occurs more specifically in breast cancer survivors than in apparent healthy individuals (55). However, it has also been reported that NK cell number and function are independent of clinical and pathological stages of the disease (9, 28). Compared with healthy individuals, NKCA was significantly decreased in women with Stage I-III breast cancer. In women with Stage IV
breast cancer, NKCA was significantly decreased compared to women with Stages I-III breast cancer (24, 34). Although a significant difference between healthy individuals and Stage I breast cancer has been reported, Contreras et al. (18) reported that NKCA was not different in Stage I breast cancer when compared to that of healthy individuals. However, in Stage II-IV breast cancer survivors, NKCA was significantly reduced in comparison with healthy individuals.

Sabbioni et al. (51) reported that the tumor load was not associated with circulating leukocyte number. However, circulating leukocyte number was associated with receptor status, with an increase in NK cell number in estrogen receptor (ER)$^+$ breast cancer survivors. Levy et al. (39) also reported that there was an increase in NKCA with ER$^-$ and progesterone receptor (PR)$^-$ receptor status when compared to ER$^+$ and PR$^+$ receptor status in breast cancer survivors. However, NKCA and ER or PR status is reported not to be correlated (24). Furthermore, HER-2/neu receptor over-expression on breast cancer cells has previously been correlated with decreased NKCA in cancer survivors when compared to those women whose tumors have lower levels of HER-2/neu receptor expression (4). HER-2 receptor over-expression in breast cancer is associated with a greater resistance to NK cell mediated lysis when compared to lower expression of HER-2 or non-expression of the HER-2/neu receptor (4, 20, 40). In summary, NK cell cytolytic function is generally reduced as cancer stage progresses and women with breast cancer are thought to be at increased risk for recurrent breast cancer, especially as the stage of disease increases.
Stress and NK Cell Function in the Breast Cancer Population

A relationship exists between psychological stress and certain immune cell functions (43). Chronically stressed individuals may exhibit a dysregulation of cytokines, elevated secretion of proinflammatory cytokines and decreased NKCA (43). Psychological and emotional stressors have been associated with the progression of disease as well as vulnerability to illness (7). Stress may affect the immune system through a combination of both neural and hormonal pathways (7). This may contribute to the etiology and progression of many disorders such as viral infections, wound healing, HIV, and cancer (7).

Neuroendocrine-immune pathways have been suggested to mediate psychosocial effects on the progression of cancer (52). Women with breast cancer are known to have higher levels of stress than healthy individuals (52). T cell cytotoxicity is compromised under conditions of chronic stress, indicating that the T cells are less effective in developing a response to defend against viruses, bacteria, and tumors (52). It has been demonstrated that people under chronic stress have a decrease in circulating T-lymphocyte number and in cancer survivors who have advanced cancer, decreases in circulating NK cell number have been observed (52). Nuclear expression of NF-κB has been demonstrated to decrease during times of psychological stress (43). It has been hypothesized that stress-induced decreases in NF-κB may decrease the secretion of cytokines that are important for an effective immune response (43).

Cancer has also been documented to result in a reduction in quality of life (QOL) related outcomes that include depression, anxiety, body image concerns, decreased self-esteem, loss of a sense of control, and social isolation (19). Although these symptoms
peak while treatment is occurring, they may last for months to years after treatment is completed (19).

Depression in cancer survivors, across all cancers, can be as high as 58% and as low as 4.5 % (1). Depression has been linked to lower proliferative response of lymphocytes to mitogens, decreased NKCA, and altered populations of circulating lymphocytes (1).

**Complementary/Alternative Medicine**

Psychosocial interventions that provide relaxation techniques training have been helpful in enhancing NK cell function, perhaps through counterbalancing the effects of stress on immunity (52). Sephton *et al.* (52) examined the relationship among spiritual expression, NKCA, and disease progression in women with stage IV metastatic breast cancer. These authors reported that women who perceived spiritual expression as more important had greater numbers of circulating NK cells and increased NKCA when compared to those women who perceived spiritual expression as less important. Levy *et al.* (39) also examined the relationship between social support and NKCA. These authors reported that increased social support among breast cancer patients resulted in greater NKCA and slower disease progression, when compared to those breast cancer patients with less perceived social support. Carlson *et al.* (13) examined the effects of an eight-week mindfulness-based stress reduction (MBSR) program in early stage breast (stage 0, I, II) and prostate (localized) cancer patients on patients’ quality of life, mood states, stress symptoms, circulating lymphocyte count and altered cytokine production of tumor necrosis factor (TNF), interferon gamma (IFN-γ), interleukin (IL)-4 and IL-10 in *in vitro*
PMA/ionomycin stimulated peripheral blood lymphocytes. The MBSR program incorporated relaxation, meditation, as well as yoga. These authors reported a decrease in symptoms of stress, including anxiety and depression, following MBSR intervention. An increased quality of life, defined by an increase in the total number of hours of sleep each night, was also reported in those survivors who completed the MBSR program. However, the MBSR intervention did not alter circulating lymphocyte subtype percentages or lymphocyte number (13).

Psychological stress alters the secretion of cytokines, with a decrease of the anti-inflammatory cytokines, such as IL-4 and an increase in the pro-inflammatory cytokines, such as IFN-γ (42). The secretion of IL-4 by T helper (Th)-2 cells acts to suppress the functions of T helper (Th)-1 cells, such as the secretion of INF-γ (42). Following MBSR intervention, stimulated lymphocytes from cancer survivors exhibited increased production of IL-4 and decreased IFN-γ production (13). Carlson et al. (13) suggested that such changes associated with MBSR were conducive with a shift from a stressed state to a less stressed and a more healthy psychological state.

The prevalence of CAM intervention is increasing among patients with life threatening diseases such as HIV or cancer (53). It has been estimated that the proportion of breast cancer survivors in the USA and Canada using CAMs ranges from 67% to 83% (53). The most prevalent forms of CAMs used among breast cancer survivors included tai chi, yoga, or chi gong because of the patients’ belief that they will reduce stress and thus have immunological benefit (53).
Demands of Illness

Demands of illness refer to the hardships or stressors that require coping or adjustment response to illness (29). Several different tools have been developed in order to empirically measure demands associated with different illnesses. These instruments have limited utility when they are restricted solely to a specific chronic disease, a specific time period during the course of the disease, or when only a unidimensional construct is measured. The “ideal” demands inventory is multidimensional, able to identify the demands associated with a variety of different chronic illnesses, and reliable across the dynamic trajectory of illness (29).

The Demands of Illness Inventory (DOII) developed by Haberman et al. (29), is one such tool of measurement that conforms to these standards. Construct validity has been established in a sample of breast cancer survivors, women with fibrocystic breast disease, and women with diabetes and all seven subscales demonstrated good internal consistency reliability ranging from 0.80 to 0.92.

The original 125-item DOII was shortened in 1989 to 59 items by deleting the family and physical symptom subscales (11). It was later revised to a 66-item inventory with two additional global measures of prior and current illness-related demands. The 68-item tool was selected for the current study to reduce responder burden. It includes five subscales: 1) personal meaning, which is the priorities, values and goals that change with the illness, 2) social relationships including changes in social activities as well as helping others deal with the illness, 3) self-image, which is changes in physical appearance or feelings of attractiveness, 4) monitoring symptoms, including vigilance to new bodily sensations, preoccupation with symptoms, fears of recurrence, or the
progressive nature of the disease, and 5) treatment issues, which includes accommodation to regimen, treatment evaluation, or the relationship with the providers.

Each item is scored on a 5-point Likert scale that ranges from 0 to 4 where 0 represents a response of “not at all” and a 4 represents a response of “very much”. All of the items on the questionnaire include the phrase “As a result of my illness….”. The tool measures frequency of 66 demands (Range 0-66) and intensity of demands (Range 0-264). Higher DOII scores are indicative of higher frequency of demands or a higher intensity of the demands of the illness (29). Two global items are rated on a 10 point Likert scale from 0 (not at all) to 10 (the worst hardship imaginable).

The DOII has previously been used to determine the demands associated with colorectal cancer patients (22, 33), breast cancer, fibrocystic breast disease, diabetes (12), and bone marrow transplantation (11). To date, the DOII has not been used to determine correlations between illness hardships and immune function.

**Breast Cancer and Medical Treatments**

The stages of breast cancer range from 0 to IV. A stage 0 diagnosis indicates that the cancer is located within the ducts, lobules or milk-producing glands, and has not spread to the surrounding fatty tissue. Stage I is assigned to tumors that are 2.0 cm or smaller in diameter and have not yet spread outside of the breast. Stage II is assigned when the tumor is larger than 2.0 cm but less than 5.0 cm and/or has spread to the ipsilateral non-fixed lymph nodes. Stage III indicates that the tumor is larger that 5.0 cm or has spread to the fixed lymph nodes, skin, chest wall, or internal mammary lymph nodes. Stage IV is assigned when the tumor has metastasized to other sites (59).
Stage IV breast cancer, or metastatic breast cancer, is generally an incurable condition (57). In the United States, over 80,000 women develop metastatic breast cancer each year (57). The mean survival time for women with metastatic breast cancer after diagnosis is 18-24 months, although this has been known to vary (57). The goal of treatment in women with metastatic breast cancer is to increase quality of life and improve survival time (57).

The human epidermal growth factor receptor (HER2) is over-expressed in 25-30% of women with breast cancer, increasing HER2 cell-surface receptor expression (56, 57, 64). HER2 is a transmembrane tyrosine kinase receptor that is involved in the control of cell growth, survival and differentiation (57), including that of tumor formation (58). Trastuzumab (Herceptin®) is a recombinant monoclonal antibody that functions to block the tyrosine kinase receptor, HER2, as well as initiate immune defense mechanisms such as the activation of macrophages and natural killer cells toward cytotoxic activity against Herceptin-bound tumor cells (58). Herceptin monotherapy for breast cancer survivors with HER2+ tumors prolongs time of disease progression; increases rate of response to treatment, prolongs the duration of response, and improves overall survival (56). Trastuzumab used in conjunction with chemotherapy, inhibits the growth of tumors in metastatic breast cancer and reduces the relative risk of death by 20% (56, 57). Herceptin is reported to act solely on the tumor; however, there is a risk of cardiomyopathy with Herceptin treatment (56). In women with HER2+ breast cancer who have been previously treated with chemotherapy, Herceptin monotherapy has a 26% response rate (64).
The functions of Herceptin are mediated by several mechanisms. Herceptin can act as an antagonist, acting as a false ligand for the over-expressed HER2 receptors. It can function to accelerate the cellular internalization and degradation of HER2. Herceptin can target abnormal cells for destruction by the cell-mediated immune system. Herceptin can also function to inhibit DNA repair mechanisms in abnormal cells (38).

Taxanes can be used in combination with Herceptin for treatment of HER2+ breast cancer. The antitumor effect of taxanes is mediated through binding to tubulin and results in microtubule stabilization, mitotic arrest and ultimately cell death (60, 62). Taxanes are also able to induce immunological effects, thereby suppressing cancer through mechanisms other than inhibition of cell division. Leukopenia, described by a decrease in neutrophil number, accompanies taxane therapy (60). Taxanes are capable of decreasing NKCA, decreasing concavalin A (ConA) induced T lymphocyte proliferation, and increasing the production of pro-inflammatory cytokines (60, 62). T-lymphocyte proliferation induced by PHA was not affected by taxane therapy (60). Taxanes have also been reported to stimulate signaling pathways such as NF-κB in pre-B cells and macrophages (37, 48).
Summary

It is well established that breast cancer survivors experience compromised immune function, often as a result of psychosocial stress. An important component of proper immune function includes the regulation of nuclear transcription factor NF-κB, which may be dysregulated with stress. NF-κB is an important regulator of lymphocyte function, including that of NK cells. NK cells are known to play an important role in innate immunity and more particularly in tumor surveillance. Recently the use of CAM’s has become very prevalent among breast cancer survivors because of its putative benefits on the immune system. However, to date no study has focused on the effects of a complementary Iyengar yoga intervention on lymphocyte transcription factor NF-κB regulation in women with breast cancer.
CHAPTER THREE

MATERIALS AND METHODS

Patient Population

Female breast cancer survivors (ages 31-57, n=10), who were patients at Cancer Care Northwest, Spokane, WA, were recruited for the study. The women were previously diagnosed with stage II-IV HER2+ breast cancer and receiving Trastuzumab (Herceptin®). Washington State University Institutional Review Board (IRB) approved the study and written consent was obtained from the women before data collection.

Participants were included in the study based upon the following inclusion criteria: A diagnosis of Stage II-III breast cancer with no evidence of disease following initial therapy or diagnosis of Stage II-III breast cancer with recurrence, followed by chemotherapy and Herceptin®, stable with minimal disease or diagnosis of Stage II-IV breast cancer but stable disease, currently receiving Herceptin® monotherapy or initial diagnosis of Stage II-IV breast cancer, currently receiving Herceptin® regime with chemotherapy or received chemotherapy and currently on Herceptin® treatment. Approved chemotherapy agents included: taxanes, carboplatin, capecitabine (Xeloda®), vinorelbine tartrate (Navelbine®). Supportive therapies such as panidronate (Aredia®), zoledronic acid (Zometa®) and leuprolide acetate (Lupron®) were allowed. Prior or concurrent radiation therapy was allowed. Scheduled radiation was permitted to be completed during the study. Other criteria included English speaking and 21 years of age or older. Surgery for a lumpectomy, mastectomy or mastectomy with reconstruction and adjuvant chemotherapy, hormonal therapy, or radiation therapy at any time in the
past was allowed. The women were required to continue on their current treatment regime during the course of the yoga intervention. Exceptions were allowed for unforeseen changes that occurred during the intervention. The women were allowed to continue the yoga intervention if their physical condition and their physician allowed. Lastly, the women were required to be physically and mentally capable of participation and willing to attend all yoga sessions.

Participants were excluded from the study based upon the following criteria: pregnant or lactating (nursing) women, cancer besides breast cancer, an active serious infection or immune disorder. Women with a documented history of psychiatric disorders requiring use of medications and women with documented alcohol or drug abuse were not permitted to participate. Women taking hormonal therapy with antiestrogen drugs (tamoxifen, or letrozole) and aromatase inhibitor (exemestane) were not permitted to participate.

Women self-selected into the yoga treatment (n=7) or treatment as usual (TAU) group (n=3). The TAU group continued to receive their regular clinical treatment. The experimental group (n=7) participated in an 8-week Iyengar yoga intervention following their doctors' release to participate in the program. The 8-week Iyengar yoga intervention program consisted of 16 instructed classes which focused on yoga philosophy, meditation techniques and various yoga poses (see Appendix B).
Preparation of PBMCs

Heparinized blood samples (~60 mL) were obtained by venipuncture or through sterile access of indwelling catheters. Peripheral blood mononuclear cells (PBMCs) were separated from whole blood by centrifugation over Histopaque separation medium (Product No. H8889, Sigma, St Louis, MO). Whole blood (15 mL) was first diluted with an equal volume of calcium-magnesium free phosphate-buffered saline (CMF) and mixed well by inversion. Histopaque (12 mL), at room temperature, was added to a 50 mL centrifuge tube and the blood-saline mixture (30 mL) was carefully layered on top of the Histopaque. The blood samples were centrifuged (CR3i, Jouan Centrifuge, S/N 400080086, LabCare America, Winchester, VA) at 400 g for 30 min at room temperature. Using a pasteur pipette, the middle opaque layer, containing the lymphocytes, was removed and transferred into a clean centrifuge tube containing 20 mL of CMF. The contents were mixed gently by inversion and centrifuged at 250 g for 10 min. The supernatant fluid was aspirated and discarded. The pellet was washed by re-suspending the cells in 20 ml of CMF and centrifuged at 250 g for 10 min. This wash process was repeated two times. Following the washes, the pellet was re-suspended in 20 ml of CMF and counted using a hemocytometer to obtain cell numbers.
**rIL-2 Activation of NF-κB**

The stimulation assay was performed in complete Roswell Park Memorial Institute (RPMI)-1640 tissue culture media (Cat No: SH30306.02, Hyclone, Logan, UT) (46). PBMCs were adjusted to a concentration of 1x10⁶ cells/ml and incubated (Water-Jacketed US Autoflow Automatic CO₂ Incubator, NuAire, Plymouth, MN) with human recombinant (r) IL-2 (1000U/ml; Cat No. 136, NIH AIDS Research and Reference Reagent Program, Rockville, MD) in 24-well flat bottom tissue culture plates (Product No. 3526, Corning Incorporated, Corning, NY) for 24 hours at 37°C and 5% CO₂ (68). After stimulation the non-adherent cells (enriched lymphocytes) were harvested using a pipette, centrifuged to collect cells, and cryopreserved in liquid nitrogen as described below.

**PHA/PMA Activation of NF-κB**

The stimulation assay was performed in complete RPMI-1640 tissue culture media (46). PBMCs were adjusted to a concentration of 2x10⁶ cells/ml and incubated with 10 µg/ml of phytohemagglutinin (PHA; Cat No: 30852701, Remel Inc, Lenexa, KS), and a 10 ng/ml phorbol 12-myristate 13-acetate (PMA; Product No. P8139, Sigma, St. Louis, MO) and 100 ng/ml Ionomycin (Cat No: 407950, Calbiochem, La Jolla, CA) in 24-well flat bottom tissue culture plates for 48 hours at 37°C and 5% CO₂. After stimulation the non-adherent cells (enriched lymphocytes) were harvested using a pipette and placed in a 50 mL centrifuge tube, centrifuged, and were prepared for cryopreservation in liquid nitrogen as described below.
Cryopreservation and Thawing Procedures

Cryopreservation of harvested lymphocytes was performed in freezing medium, composed of 50% Fetal Bovine Serum (FBS; Cat No. SFB30, Equitech-Bio Inc, Kerrville, TX), 40% complete RPMI-1640 media and 10% Dimethyl Sulphoxide (DMSO; Product No. D2650, Sigma, St. Louis, MO) (17). Non-adherent, enriched lymphocytes were harvested, centrifuged for 10 min at 400 g and re-suspended in cold (4°C) freezing media at a concentration of 5x10^6 cells/ml. Cells (1 mL) were placed in 1.8 ml cryovials and frozen by storing them at 4°C for 30 min, subsequently stored at 0°C for one hour, followed by storage at -80°C for four hours and finally transferred to liquid nitrogen for storage (66).

Thawing of the lymphocytes involved placing the vials into a 37°C water bath. Once the last ice crystal melted, cells were put on ice and washed two times with CMF (4 mL, 2-4°C) (17, 66). Thawed lymphocytes were further used for protein extractions, protein determination, and determination of NF-κB.

Protein Extractions

Cytosolic and nuclear extractions were performed according to the manufacturer's specifications (NE-PER® Nuclear and Cytoplasmic Extraction Reagents, Product No: 78833, Pierce Biotechnology, Rockford, IL). Briefly, in a 1.5 mL microcentrifuge tube, cells were re-suspended in CMF (500 µl), and centrifuged in a microcentrifuge (Hermle Z180 M Microcentrifuge, S/N Z111131, National Labnet Co, Edison, NJ) for 5 min at 500 g. The supernatant fluid was removed and discarded using a pasteur pipette and the
remaining cell pellet volume was estimated. Ice cold cytoplasmic extraction reagent I (CER I) containing 10 µl/ml of Halt™ Protease Inhibitor Cocktail (Product No. 78410, Pierce Biotechnology, Rockford, IL) and 10 mM sodium orthovanadate (Cat No: 567540, Calbiochem, La Jolla, CA) was added to the cell pellet at a ratio of 100µl/10µl estimated cell volume. The tube was vortexed vigorously for 15 sec and incubated on ice for 10 min. Following the 10 min incubation, ice-cold CERII was added to each tube at a ratio of 5.5µl/10µl estimated cell volume. The sample was vortexed vigorously for five sec and incubated on ice for one min. Following the incubation the tube was once again vortexed for five sec and centrifuged in a microcentrifuge for five min at maximum speed (~16 000 g). The supernatant fluid, containing the cytosolic extract, was immediately transferred to pre-chilled microcentrifuge tubes. The remaining insoluble pellet was re-suspended in nuclear extraction reagent (NER) at a ratio of 50 µl/10 µl estimated cell volume, containing 10 µl/ml of Halt™ Protease Inhibitor Cocktail and 10 mM sodium orthovanadate. The tube was vortexed vigorously for 15 sec. The tube was incubated on ice for 40 min, vortexing for 15 sec every 15 min. Following the incubation the tubes were centrifuged at maximum speed (~16 000 g) in a microcentrifuge for 10 min. The supernatant fluid containing the nuclear or cytosolic extracts were dialyzed to remove excess salts which would interfere with further assays. Briefly, supernatant fluid for cytosolic and nuclear extracts were transferred into pre-chilled dialyzing units (Slide-A-Lyzer® MINI Dialysis Units, Product No. 69570, Pierce Biotechnology, Rockford, IL) and were allowed to dialyze in CMF (0.1 M, 150 mM NaCl) overnight. Dialyzed supernatant fluid was removed from the dialyzing units and placed in a pre-chilled
microcentrifuge tubes. Nuclear and cytosolic samples were stored at -80°C until further use.

**Determination of Protein Concentration**

The protein concentrations of cytosolic and nuclear extract samples were determined according to the bicinchoninic acid (BCA) protein assay protocol (Micro BCA™ Protein Assay Kit, Product No. 23235, Pierce Biotechnology, Rockford, IL). Bovine Serum Albumin (BSA) standards were prepared in CMF according to the manufacturers instructions in order to obtain BSA concentrations of 0.5, 1, 2.5, 5, 10, 20, 40, and 200 µg/ml. The BCA working reagent (WR) was prepared by mixing 25 parts of the Micro BCA™ Reagent MA and 24 parts Reagent MB with one part of Reagent MC (150 µl WR/sample). Standards (150 µl) and unknown samples (3 µl sample + 147 µl CMF) were added in duplicate to a micro well plate. The WR (150 µl) was added to each well and the plate was mixed on a plate shaker for 30 sec. The plate was covered and incubated for 30 min at 37°C. Once the plate was at room temperature, the absorbance was read at 562 nm on the spectrophotometer (Spectra max plus, S/N MN 02696, Molecular Devices, Sunnydale, CA). The protein concentration for each unknown was calculated. This procedure is able to detect protein in samples ranging from 0.5-20 µg/ml.

**Determination of NF-κB**

Nuclear and cytosolic NF-κB was determined using a chemiluminescence ELISA assay according to manufacturers instructions (EZ-Detect™ NF-κB p65 Transcription Factor Kit, Product No. 89859, Pierce Biotechnology, Rockford, IL). Sensitivity of the is
assay for total protein is 0.12 µg, with a linear range from 0.12 µg to 10 µg; and for nuclear protein is 0.12 µg, with a linear range from 0.12 µg to 7 µg. Prepared Working Binding Buffer (50 µl) was added to each well followed by the competitor duplex to the appropriate wells. From the determined protein concentrations of each sample, nuclear protein (5 µg) or cytosolic protein (10 µg) was loaded in duplicate into each well. The plates were incubated with mild agitation for one hour at room temperature. Following the incubation, the contents were emptied and the plate was tapped dry (five times) on paper towel. The plate was washed three times with 1X Wash Buffer (200 µl) and tapped dry (five times) on paper towel between each wash. Primary antibody (100 µl) was added to each well and the plates were incubated for one hour at room temperature, without agitation. The plate contents were emptied, tapped dry on paper towel and washed three times with 1X Wash Buffer (200 µl). Secondary antibody (100 µl) was added to each well and the plates were incubated for one hour at room temperature, without agitation. The plate contents were emptied, tapped dry (five times) on paper towel and washed four times with 1X Wash Buffer (200 µl). Luminol substrate working solution (100 µl) was added to each well and immediately read using microbeta plate reader (1450 Microbeta Wallac Jet Liquid Scintillation and Luminescence Counter, S/N 4502011, Perkin Elmer life science, Boston, MA). Nuclear NF-κB was calculated by subtracting baseline values for each sample. A limitation of this assay is that the amount of bound versus unbound NF-κB cannot be detected, therefore it is unable to distinguish between the amount of free NF-κB or the NF-κB bound to IκB or the κB binding site.
Statistical Analyses

All data was analyzed using the Statistical Package for the Social Sciences (SPSS), version 11.0 for Windows. Kolomogorov-Smirnov test for normality was performed on all data. Chi-square tests were performed to determine nominal differences between groups at both time periods. One-way analysis of variance (ANOVA) with repeated measures was used to determine overall group by time effects of the yoga intervention on NF-κB measures and psychosocial data. Independent t-tests and Mann Whitney U tests were used to analyze the effects of the yoga intervention on nuclear NF-κB and psychosocial data pre-and post-intervention. After visual inspection of the data, simple linear regression analyses were performed to examine the relationships between NF-κB and selected psychosocial outcomes. Pearson product moment correlations were determined to quantify the strength of the linearly related variables. Significance for all statistical tests was established at the 0.05 level of probability. A trend was declared if: 0.05 ≤ p ≤ 0.08.


LYMPHOCYTE NF-κB AND DEMANDS OF ILLNESS MEASURES FROM WOMEN WITH STAGE II-IV BREAST CANCER DURING A COMPLEMENTARY YOGA INTERVENTION

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ABSTRACT

Breast cancer survivors are challenged by many psychosocial issues that increase the burden of survivorship and overall stress. Increased psychosocial distress alters nuclear expression of NF-κB, a regulator of gene transcription activities involved in innate immunity. Interventions that target psychosocial stressors may attenuate psychosocial distress and benefit immunity. The purpose of this study was to examine the impact of an 8-week Iyengar yoga intervention on psychosocial stressors and nuclear expression of NF-κB in rIL-2 and PHA/PMA stimulated lymphocytes of women with breast cancer. Female subjects, previously diagnosed with stage II-IV HER-2⁺ breast cancer and receiving Herceptin®, volunteered to join yoga (n=7) or treatment as usual (n=3) group. Lymphocytes obtained pre-(T₁) and post-(T₂) intervention were stimulated with rIL-2 or PHA/PMA. Cytosolic (C) and nuclear (N) extracts were analyzed for NF-κB by luminescence. Demands of Illness Inventory (DOII) measured hardships or psychosocial stressors at T₁ and T₂. Data were analyzed by ANOVA with repeated measures, independent t-tests or Mann Whitney U tests. Because clinical treatment changes were required for the management of disease, the effects of yoga on lymphocyte NF-κB stimulation index was not discernable. NF-κB stimulation index was indirectly correlated with nuclear NF-κB expression in unstimulated lymphocytes (rIL-2 stimulation index, R²=0.817, p< 0.001; PHA/PMA stimulation index, R²=0.455, p=0.016), independent of group and time. This relationship indicated that greater nuclear expression of NF-κB occurred in those lymphocytes having lower baseline NF-κB nuclear expression. Among all subjects, NF-κB N:C ratio in unstimulated lymphocytes was indirectly associated with hardships associated with breast cancer and its treatment.
that impact personal meaning (T₁: \(R^2=0.4134, p=0.045\)). PHA/PMA stimulation index of NF-κB was indirectly associated with overall DOII scores (\(R^2=0.4232, p=0.042\)), and hardships associated with monitoring treatment and disease related symptoms (\(R^2=0.772, p=0.032\)). These relationships indicated that those responders reporting increased hardships due to breast cancer disease also had lower stimulation index of lymphocyte NF-κB. The biological relevance of these results requires further investigation; however, these findings lend support to the hypothesis that NF-κB regulation and illness-associated stressors are inter-related. Further explication of the complex interplay of both types of variables could benefit breast cancer survivors.

**Key Words:** yoga, complementary medicine, breast cancer, NF-κB, immune function, psychoneuroimmunology
INTRODUCTION

Psychological, psychosocial and emotional stressors have been associated with disease progression and vulnerability to illnesses (4). Psychosocial stress may contribute to the etiology and progression of many pathologies such as cancer (4). Breast cancer survivors with advanced disease are challenged by many psychosocial issues that increase the burden of survivorship and overall stress. Women with breast cancer report chronic fear of recurrence, uncertainty, and difficulty with social adjustment (9, 24). These issues contribute to their perceived stress.

Several different tools have been developed in order to empirically measure demands associated with different illnesses. The Demands of Illness Inventory (DOII) developed by Haberman et al. (13), is one such tool of measurement. Demands of illness refer to the hardships or stressors that require coping or adjustment response to illness (13). The original 125-item DOII was shortened in 1989 to 59 items by deleting the family and physical symptom subscales (6). It was later revised to a 66-item inventory with two additional global measures of prior and current illness-related demands. The 68-item tool was selected for the current study to reduce responder burden. It includes five subscales 1) personal meaning, 2) social relationships, 3) self-image, 4) monitoring symptoms, and 5) treatment issues.

Chronic stress is associated with reduced immune responses, which may contribute to a less effective ability to defend against tumors (4). For example, persons with advanced cancer have fewer circulating natural killer (NK) cells than do healthy individuals (4). Survivors of metastatic melanoma are also reported to have defects in perforin-dependent natural killer cell activity (NKCA) and phytohemagglutinin (PHA)-
stimulated proliferation (14). Shevde et al. (26) observed that families with high incidence of cancer, particularly breast cancer, had lower than normal NK and T cell cytotoxic functions. Breast cancer survivors may have decreased circulating NK cell number and function, as well as impaired T-lymphocyte function (27, 29). NK cells play an important role in innate immunity against tumor metastasis and growth in a variety of cancers, including breast cancer (32). In vitro, tumor cytolysis by NK cells is conventionally measured by NKCA. Compromised NKCA has been associated with both the development and progression of cancer (20, 27). Therefore, women with breast cancer are thought to be at increased risk for recurrent breast cancer (20, 27).

An increasing body of evidence indicates that interventions that target psychosocial issues benefit certain immune responses, particularly NK cell function, through counterbalancing the effects of stress on immunity and slowing disease progression. Enhancement of NKCA is dependent, in part, on the cell’s inherent response to activation. NK cell stimulation by interleukin-2 (IL-2) induces activation of nuclear factor kappa B (NF-κB) proteins, leading to increased NKCA (37). In-vitro, phytohaemagglutinin, phorbol myristate acetate (PMA) and ionomycin can also be used to stimulate peripheral blood lymphocytes, including NK cells (19).

NF-κB proteins are members of the NF-κB/Rel family of transcription factors in lymphocytes. They have a central role in regulating innate immunity, primarily through the activation of genes encoding for regulators of cell proliferation and apoptosis. The NF-κB/Rel family of transcription factors consists of homo- and heterodimers of five different members of the Rel family (7, 10). These include NF-κB1 (p105/50), NF-κB2 (p100/52), RelA (p65), RelB and c-Rel (7, 10-12).
NF-κB proteins are sequestered in the cytoplasm in an inactive form due to their association with inhibitor protein IκB (2, 7, 11, 12). Activation of the NF-κB involves receptor-mediated phosphorylation and the subsequent phosphorylation-dependent ubiquitinylation of IκB, resulting in the dissociation of NF-κB from IκB (7, 11, 12). NF-κB then translocates to the nucleus and binds to specific κB sites located in the promoter region of genes, thereby regulating transcriptional activities of genes involved in specific immune responses (3, 7, 11).

Decreased NF-κB nuclear expression was observed in PHA/PMA stimulated lymphocytes of women scheduled for a breast biopsy, a condition of increased psychosocial distress (19). Based on these initial findings, the authors suggested that psychosocial stress may lead to decreased ability to activate NF-κB in lymphocytes.

Psychosocial interventions are reported to enhance certain immune responses through counterbalancing the effects of stress. The prevalence of complementary interventions is increasing among individuals with life threatening diseases, such as cancer (25). Currently, yoga is one of the most prevalent forms of complementary interventions used among breast cancer survivors as a means to provide immunological benefits (25). To date, no evidence exists regarding the effects of a complementary yoga intervention on the activation of NF-κB in lymphocytes from women with breast cancer. The purpose of this study was to examine the impact of an 8-week yoga intervention on rIL-2 and PHA/PMA stimulated nuclear NF-κB expression in peripheral blood lymphocytes of women with stage II-IV HER2+ breast cancer. The hypotheses of the study were that 1) yoga intervention would increase stimulated lymphocytes nuclear expression of NF-κB compared with breast cancer survivors who received treatment as
usual (TAU); 2) yoga intervention would decrease DOII scores; and 3) nuclear NF-κB expression would be negatively correlated with DOII scores.
MATERIALS AND METHODS

Patient Population

Female breast cancer survivors (ages 31-57), previously diagnosed with stage II-IV HER2+ breast cancer and receiving Trastuzumab (Herceptin®) were recruited for the study. Participants were included in the study based upon the following criteria: A diagnosis of Stage II-III breast cancer with no evidence of disease following initial therapy or diagnosis of Stage II-III breast cancer with recurrence, followed by chemotherapy and Herceptin®, stable with minimal disease or diagnosis of Stage II-IV breast cancer but stable disease, currently receiving Herceptin® monotherapy or initial diagnosis of Stage II-IV breast cancer, currently receiving Herceptin® regime with chemotherapy or received chemotherapy and currently on Herceptin® treatment. Approved chemotherapy agents included: taxanes, carboplatin, capecitabine (Xeloda®), vinorelbine tartrate (Navelbine®). Supportive therapies such as pamidronate (Aredia®), zoledronic acid (Zometa®) and leuprolide acetate (Lupron®) were allowed. Prior or concurrent radiation therapy was allowed. Scheduled radiation was permitted to be completed during the study. Other criteria included English speaking and 21 years of age or older. Surgery for a lumpectomy, mastectomy or mastectomy with reconstruction and adjuvant chemotherapy, hormonal therapy, or radiation therapy at any time in the past was allowed. The women were required to continue on their current treatment regime during the course of the yoga intervention. Exceptions were allowed for unforeseen changes that occurred during the intervention. The women were allowed to continue the yoga intervention if their physical condition and their physician allowed.
Lastly, the women were required to be physically and mentally capable of participation and willing to attend all yoga sessions.

Participants were excluded from the study based on any of the following criteria: pregnant or lactating (nursing); diagnosis of cancer other than breast cancer; an active serious infection or immune disorders; a documented history of psychiatric disorders requiring use of medications; documented alcohol or drug abuse; use of hormonal therapy with antiestrogen drugs (tamoxifen, or letrozole) and aromatase inhibitor (exemestane); and use of steroids or any drugs that are known to influence the immune system.

Women self-selected into the yoga (n=7) intervention or treatment as usual (TAU) (n=3). The TAU group continued to receive their regular clinical treatment. The yoga group participated in an 8-week Iyengar yoga intervention following their doctors' release to participate in the program. The 8-week Iyengar yoga intervention consisted of 16 instructed classes which focused on yoga philosophy, meditation techniques and various yoga poses (see Appendix B). Yoga was practiced two times per week under class instruction in addition to one time each week of home self instruction. Washington State University Institutional Review Board (IRB) approved the study and written consent was obtained from the women before data collection.

Psychosocial Analysis

Demand of Illness Inventory (DOII) was used to measure the burden of psychosocial stressors prior to (T₁) and following intervention (T₂). The 68-item tool was selected for the current study to reduce responder burden. It included five subscales 1) personal meaning, which is the priorities, values and goals that change with the illness, 2) social relationships including changes in social activities as well as helping others deal
with the illness, 3) self-image, which is changes in physical appearance or feelings of attractiveness, 4) monitoring symptoms, including vigilance to new bodily sensations, preoccupation with symptoms, fears of recurrence, or the progressive nature of the disease, and 5) treatment issues, which includes accommodation to regimen, treatment evaluation, or the relationship with the providers.

Each item is scored on a 5-point Likert scale that ranges from 0 to 4 where 0 represents a response of “not at all” and a 4 represents a response of “very much”. All of the items on the questionnaire include the phrase “As a result of my illness….”. The tool measures frequency of 66 demands (Range 0-66) and intensity of demands (Range 0-264). Higher DOII scores are indicative of higher frequency of demands or a higher intensity of the demands of the illness. Two global items are rated on a 10 point Likert scale from 0 (not at all) to 10 (the worst hardship imaginable) (6, 13).

Preparation of PBMCs

Heparinized blood samples from subjects were obtained by venipuncture or through sterile access of indwelling catheters within one week prior to (T1) and following (T2) intervention. Peripheral blood mononuclear cells (PBMC) were separated from whole blood by centrifugation over Histopaque separation medium (Sigma, St Louis, MO). The pellet was re-suspended in phosphate-buffered saline (CMF) and cell counts were obtained from re-suspended samples.
rIL-2 Activation of NF-κB

The stimulation assay was performed in complete RPMI-1640 tissue culture media (21). PBMCs were adjusted to a concentration of $1 \times 10^6$ cells/ml and incubated with rIL-2 (1000U/ml; NIH Aids Research and Reference Reagent Program, Rockville, MD) in 24-well flat bottom tissue culture plates for 24 hours at 37°C and 5% CO₂ (37). After stimulation the cells were harvested and cryopreserved (8, 36).

PHA/PMA Activation of NF-κB

The stimulation assay was performed in complete RPMI-1640 tissue culture media (21). PBMCs were adjusted to a concentration of $2 \times 10^6$ cells/ml and incubated with 10 ug/ml of PHA (Remel Inc, Lenexa, KS), 10 ng/ml of PMA (Sigma) and 100 ng/ml ionomycin (Calbiochem, La Jolla, CA) in 24-well flat bottom tissue culture plates for 48 hours at 37°C and 5% CO₂. After stimulation the cells were harvested and cryopreserved (8, 36).

Determination of NF-κB

Cytosolic and nuclear extractions were performed according to the manufacturer's specifications (Pierce Biotechnology, Rockford, IL). Cytosolic and nuclear extracts were dialyzed in CMF overnight. Samples were stored at -80°C until further use.

The protein concentrations of cytosolic and nuclear extract were determined according to the bicinchoninic (BCA) protein assay protocol (Pierce Biotechnology, Rockford, IL). Bovine Serum Albumin (BSA) standards were prepared according to the
manufacturer’s instructions. Standards and unknown samples were added in duplicate to a microwell plate. Absorbance was read at 562 nm (Spectra max plus, Molecular Devices, Sunnydale, CA). The protein concentration for each unknown sample was calculated.

Cytosolic (10 µg) and nuclear (5 µg) protein extracts were analyzed in duplicate for NF-κB by a chemiluminescence ELISA assay according to the procedures set forth by PIERCE (EZ-Detect Transcription Factor Kit, Pierce, Rockford, IL), using 1450 Microbeta Wallac Luminescence Counter (Perkin Elmer Life Science, Boston, MA).

Stimulation index, a marker of increased nuclear NFκB expression, was calculated by the following formula:

\[
\frac{(\text{nuclear NF-κB post-stimulation}) - (\text{nuclear NF-κB pre-stimulation})}{(\text{nuclear NF-κB pre-stimulation})} \times 100\%
\]

Statistical Analyses

All data was analyzed using the Statistical Package for the Social Sciences (SPSS), version 11.0 for Windows. Kolomogorov-Smirnov test was used to test data for normality. Chi-square tests were performed on nominal data. One-way analysis of variance (ANOVA) with repeated measures was used to determine overall group, time, and group by time interaction effects on NF-κB measures and psychosocial data. Independent t-tests or Mann-Whitney U tests were used to analyze the effects of the yoga intervention on nuclear NF-κB and psychosocial data pre- and post-intervention. After visual inspection of the data, simple linear regression analyses were performed to examine the relationships between NF-κB and selected psychosocial outcomes. Pearson
product moment correlations were determined to quantify the strength of the linearly related variables. Significance for all statistical tests was established at the 0.05 level of probability. A trend was declared if: $0.05 \leq p \leq 0.08$. 
RESULTS

Demographic data, medical history, treatment history, stage of cancer and other variables are presented in Table 1. The ages of the yoga (n=7) and TAU (n=3) breast cancer survivors ranged from 38-55 years and 44-55 years, respectively. Twenty-nine percent of the breast cancer survivors in the yoga group were diagnosed with Stage II, 14% with Stage III and 57% with Stage IV breast cancer. In TAU, 33% of the breast cancer survivors were diagnosed with Stage III and 67% with Stage IV breast cancer. In the yoga group, 57% of the breast cancer survivors were classified as ER+/PR+ receptor status and 43% of the breast cancer survivors were ER-/PR- receptor status. Thirty-three percent of the breast cancer survivors in the TAU were classified as ER+/PR+ receptor status and 67% of the breast cancer survivors were ER-/PR-. The percentage of breast cancer survivors diagnosed with breast cancer for one year or longer was 71% and 67% in the yoga and TAU groups, respectively. In the yoga group, 43% were currently undergoing chemotherapy treatment while 67% of TAU group were currently undergoing chemotherapy treatment.

There were no significant group, time, or time by group interaction effects for lymphocyte nuclear NF-κB expression in PHA/PMA stimulated or in rIL-2 stimulated cells. However, a significant time effect (p=0.014) and a non-significant time by group interaction (p=0.081) were observed for nuclear to cytosolic (N:C) ratio of NF-κB in unstimulated lymphocytes. The yoga group had a greater lymphocyte NF-κB N:C ratio in unstimulated lymphocytes at T2 than did the TAU group.
A 60% response rate was observed for rIL-2 and PHA stimulation leading to increased nuclear expression of NF-κB in lymphocytes. These samples were designated as NF-κB responders (Table 1). Those lymphocytes that had less nuclear NF-κB expression following stimulation as compared with that of unstimulated lymphocytes were designated as non-responders. There was no significant group difference in the response rate to rIL-2 stimulation at T₁ (χ²=1.27, p=0.260) and T₂ (χ²=0.079, p=0.778) or to PHA/PMA stimulation at T₁ (χ²=0.79, p=0.667) and T₂ (χ²=0.79, p=0.667). To evaluate whether response rates differed from that of healthy subjects, lymphocytes were collected from two apparently healthy volunteers and analyzed for rIL-2 and PHA/PMA stimulation index. The response rates of cells from breast cancer survivors did not differ from that of healthy subjects at T₁ (χ²=2.571, p=0.276) and T₂ (χ²=1.286, p=0.526) for rIL-2 stimulation or at T₁ (χ²=0.526, p=0.526) and T₂ (χ²=0.526, p=0.526) for PHA stimulation.

Initially, data from those samples responsive to stimulation were evaluated blinded to the changes in clinical treatment required for management of the participants’ disease. There was a significant group effect (p < 0.001) for NF-κB N:C ratio in unstimulated lymphocytes. NF-κB N:C ratio was lower in the yoga group when compared to the TAU group. Significant time by group interactions were observed for stimulation index of NF-κB in cells responsive to rIL-2 stimulation. The yoga group exhibited a greater stimulation index versus the TAU group at T₂ (p=0.015, Fig 1A).

In lymphocytes responsive to PHA/PMA stimulation, no significant group, or time by group interaction effects were observed for stimulation index of NF-κB. There was a significant time effect (p=0.028, Fig 1B). There was also a significant increase
in stimulation index of NF-κB in PHA/PMA stimulated lymphocytes of the responders in the yoga group. Nuclear to cytosolic (N:C) ratio of NF-κB in unstimulated lymphocytes did not change significantly, although there was a trend (p=0.084) at T2 for a lower N:C ratio of NF-κB in the yoga group as compared to that of the TAU group.

Comparisons were made between responders and non-responders to evaluate whether baseline nuclear expression of NF-κB in unstimulated lymphocytes influenced the stimulation response to rIL-2 or PMA/PHA. No significant differences were observed between the responders and non-responders for nuclear NF-κB expression or N:C ratio of NF-κB in unstimulated lymphocytes at T1 or T2. This indicated that baseline nuclear NF-κB expression was not related to changes in nuclear NF-κB expression following stimulation. There were no significant differences in lymphocyte N:C NF-κB ratio or in stimulation index between cells stimulated with rIL-2 or PHA/PMA.

In reverse relationships were observed between the amount of nuclear NF-κB in unstimulated cells and rIL-2 stimulation index (\(R^2=0.817, p<0.001\), Fig.2A) and PHA/PMA stimulation index (\(R^2=0.455, p=0.016\), Fig.2B), independent of group. An inverse relationship was also observed between N:C ratio of NF-κB in unstimulated cells and rIL-2 stimulation index (\(R^2=0.427, p=0.029\), Fig. 3A) and PHA/PMA stimulation index (\(R^2=0.388, p=0.031\), Fig. 3B), independent of group. Baseline nuclear NF-κB expression and baseline N:C ratio of NF-κB were directly associated (\(R^2=0.3215, p=0.009\), Fig.4). This is an indication of how accurately nuclear NF-κB reflects the distribution of NF-κB across the cell. The rIL-2 stimulation index of NF-κB was also
directly associated with PHA/PMA stimulation index of NF-κB ($R^2=0.5699$, $p<0.001$, Fig.5). This indicates that the cells response to either stimulation procedure was similar.

Three of the yoga subjects were removed from statistical analyses for the following reasons. One subject completed chemotherapy treatment between $T_1$ and $T_2$. One subject treated with dexamethasone at $T_1$ and not at $T_2$. A third subject treated with dexamethasone two days prior to $T_1$ and 14 days prior to $T_2$. Data were re-analyzed using Mann-Whitney U test. No differences were observed between yoga ($n=2$) and TAU ($n=2$) groups for rIL-2 stimulation index ($p=0.439$, $p=0.121$) or for PHA/PMA stimulation index ($p=0.667$, $p=0.667$).

Changes in chemotherapy can alter NF-κB expression (28). Therefore, the effect of chemotherapy on lymphocyte nuclear NF-κB expression was investigated in the presented study. Chemotherapy treatment was associated with a non-significant ($p=0.085$) increase in nuclear NF-κB expression in unstimulated lymphocyte samples at $T_1$ and $T_2$ ($n=10$).

**Psychosocial Factors**

Data from DOII measures of different subscales are presented in Table 2. Significant time ($p=0.010$) and group by time interaction ($p=0.002$; Fig. 6) effects were observed for hardships associated with breast cancer and treatment related to personal meaning. Significant time ($p=0.047$) and group by time interaction ($p=0.039$; Fig. 7) effects were observed for the hardships imposed by breast cancer and treatment related to social relationships. The yoga group had a decrease in DOII scores for hardships associated with breast cancer and treatment related to personal meaning at $T_2$, when
compared to the TAU group. The yoga group also had an increase in DOII scores for the hardships imposed by breast cancer and treatment related to social relationships at T2, when compared to the TAU group. All other psychosocial factors remained stable pre- and post-intervention. There findings indicate that this sample of breast cancer survivors were relatively stable for the psychosocial subsets measured.

Associations among the various psychosocial outcomes and NF-κB measures were investigated, first by exploring those relationships among all subjects and second, among only those subjects whose lymphocytes responded to stimulation with rIL-2 or PHA/PMA. Among all subjects, the NF-κB N:C ratio in unstimulated lymphocytes was inversely associated with the hardships associated with breast cancer and its treatment; specifically those hardships that were perceived to impact personal meaning in life ($R^2=0.4134$, $p=0.045$ at $T_1$, Fig. 8 and $R^2=0.570$, $p=0.083$ at $T_2$). PHA/PMA stimulation index at $T_1$ was inversely associated with the overall DOII scores at $T_1$ ($R^2=0.4232$, $p=0.042$; Fig. 9).

Among the rIL-2 responders, stimulation index of lymphocyte NF-κB was inversely associated with the global perception of current hardship imposed by breast cancer ($R^2=0.353$, $p=0.070$), independent of time or group effects. Stimulation index of lymphocyte NF-κB was inversely associated with the global perception of illness and treatment hardships since the time of original diagnosis ($R^2=0.697$, $p=0.078$) at $T_2$.

Among the PHA/PMA responders, stimulation index of NF-κB was inversely associated at $T_1$ with monitoring treatment and disease related symptoms ($R^2=0.772$, $p=0.032$; Fig. 10). An inverse association was also observed for the global perception of illness and treatment hardship since the time of original diagnosis and stimulation index
of NF-κB (R²=0.3069, p=0.077), independent of group and time effects. Overall, these relationships indicated that nuclear NF-κB stimulation index was greater in lymphocytes from women who reported fewer demands of illness.
DISCUSSION

Cancer survivors often have compromised immune function at varying levels and degrees. One area of importance in proper immune function is the ability of T cells to respond to stimulation and translocate signals to the nucleus of the cell. Stimulation of peripheral blood lymphocytes from breast cancer survivors with rIL-2 and PHA/PMA did not result in detectable increases in nuclear expression of NF-κB in all samples. As previously indicated, only 60% of the samples were responsive to rIL-2 and PHA/PMA stimulation. There are several different hypotheses that could explain these observations. One possibility is that there might have been an error in the cell signaling pathway in the non-responsive subjects. Jovic et al. (14) reported that lymphocytes from metastatic melanoma patients were unresponsive to PHA stimulated proliferation, possibly due to an error in the cell signaling pathway. There are numerous studies that indicate an error in T cell signaling pathway among cancer patients (15-17, 33). As cancer progresses, there has been reported to be an increase in cell signaling defects (33). Survivors of metastatic melanoma have defects in T cell signaling (17). T cells of cancer patients may have altered phosphorylation and kinase activity, such as a reduction of phosphorylated substrates, a decrease in the duration of phosphorylation of substrates and a decrease in protein tyrosine kinase (PTK) synthesis (15, 17). All of the above components are important components for cell signaling pathways that lead to activation of transcription factors, such as NF-κB (17). Transcription factors, such as NF-κB control the expression of many components of immune molecules, including IL-2R α, IL-2, TCR β, MHC I, MCH II, and TNF-α (17). In a recent study, Kurt et al. (16) reported that 29% of the breast cancer patients in their study had a defect in T cell signaling, presenting as a
decrease in cell surface expression of T cell receptor-zeta (TCR-ζ). Defects of the cell signaling pathway in T cells or NK cells can lead to a variety of immunological consequences, including changes in cytokine production, decreased proliferative responses and decreased cytotoxic functions.

Decreased expression of NF-κB in the T cells of individuals with cancer has been reported (17). Defects in Rel-A (p65) were observed in 38% of breast cancer patients as reported by Li et al. (18). These results lend to support the possibility of an impaired stimulation response in lymphocytes of breast cancer survivors. This may suggest that 40% of the breast cancer survivors in our study might have had defects in T cell signaling, resulting in NF-κB remaining sequestered in the cytosol.

A second possibility as to the undetectable stimulation in 40% of the breast cancer survivors may be due to the transient nature of NF-κB. Prolonged stimulation of lymphocytes and the increased nuclear NF-κB can result in the up-regulation of IκB synthesis. It has been proposed that NF-κB may cause the transcriptional activation of IκBα (1, 31). The cytosolic inhibitory protein can be recovered within one hour. The increased synthesis of IκBα results in increased cytosolic IκBα expression. IκB can enter the nucleus, interact with NF-kB and therefore inhibit its association with the κB binding site (1, 31). NF-κB, attached to its inhibitory protein, exits the nucleus, resulting in more NF-κB being sequestered in the cytosol. The re-sequestered NF-κB into the cytosol may explain the lack of stimulation observed in lymphocytes of some of the breast cancer survivors in our study.

It is also of interest to note that the response of the cell to rIL-2 and PHA/PMA stimulation was, in part, dependent upon initial levels of nuclear NF-κB in unstimulated
cells. Lymphocytes with greater nuclear NF-κB before stimulation were less responsive to stimulation by rIL-2 and PHA/PMA. This indicates that there may be a ceiling effect to the amount of NF-κB that can be present within the nucleus at a given time. This ceiling effect may, in part, explain the lack of response to rIL-2 and PHA/PMA stimulation of NF-κB in 40% of the lymphocytes from the breast cancer survivors in our study. Unstimulated lymphocytes with a greater amount of nuclear NF-κB, also had a lower stimulation indexes when compared to those lymphocytes with lower amounts of nuclear NF-κB. It is possible that these cells may have been already maximally stimulated by some other mechanism and thus, there was no observed effect with rIL-2 or PHA/PMA stimulation of NF-κB.

It is not possible at this point in time to report that a yoga intervention alters nuclear NF-κB in lymphocytes of women with stage II-IV HER-2+ breast cancer. As previously indicated, three of the yoga subjects were removed from statistical analysis. One of the yoga subjects was undergoing chemotherapy treatment at T₁ and not at T₂. Chemotherapy treatment results in suppressed immune responses defined as a decreased NKCA which continually increases and returns to normal at least six months post treatment (5). Treatment with chemotherapy may also result in chronically increased activation of NF-κB (30), which can lead to an upregulation of IκB synthesis. This would result in less NF-κB being translocated to the nucleus upon stimulation. Medications, such as dexamethasone, were also being used by two of the yoga subjects at T₁ and not T₂. Dexamethasone is a gluccocorticoid that may interfere with NF-κB activation. Gluccocorticoids act to inhibit the activation of NF-κB through different mechanisms. Hypothesized mechanisms are that gluccocorticoids may function to inhibit
NF-κB activation are that they 1) upregulate the expression of IκB, 2) block the κB binding site on DNA, 3) form a complex with NF-κB, or 4) compete with NF-κB for co-activators (2). These three subjects discussed above had an increase in nuclear NF-κB, as well as an increase in stimulation index of NF-κB at T₂ in comparison to T₁. However, because these subjects’ clinical treatment regime did not remain constant from T₁ to T₂, it is not possible to determine if the increase in NF-κB was due to yoga or a change in clinical treatment. Other factors such as chemotherapy treatment and dexamethasone may have interfered with the results. Of the remaining two subjects in the yoga group, there was a trend toward an increase in stimulation index of NF-κB, although this was not significant. However, this study does warrant further investigation with a larger sample size and with the inclusion of subjects on stable treatment.

Another issue worth consideration is the effect of morphine on NF-κB activation. One of the breast cancer survivors in yoga group was using morphine and was a non-responder to both rIL-2 and PHA/PMA stimulation of NF-κB. Morphine has been reported to impair macrophage, neutrophil, and monocyte function by interfering with the binding of NF-κB to the κB binding site in the nucleus (22, 34, 35). Macrophages pretreated with morphine (50 mM) and activated with LPS (10 µg/ml, 24 hr) had a decrease in nuclear binding of the p50 p65 NF-κB heterodimer (22). It has been hypothesized that morphine may increase IκB in the cytosol by inhibiting the phosphorylation of IκB, increasing the dephosphorylation of IκB, increasing the synthesis of IκB or by decreasing the degradation of IκB (22). Welters et al. (34, 35) reported that morphine causes the release of nitric oxide (NO), which directly inhibits DNA binding
activity of NF-κB in TNF-α stimulated neutrophils. Therefore, it is possible that this breast cancer survivor was a non-responder due to the effects of morphine.

The relationship between psychosocial stress and immune function has been examined (4, 19). Stressed individuals often present with cytokine dysregulation, decreased NK cell and T cell number in peripheral blood, decreased NKCA and decreased T cell function (4, 19, 23, 29). The psychosocial stress experienced by women prior to a breast biopsy was associated with decreased nuclear NF-κB in lymphocytes (19). NF-κB is of particular importance in that it regulates the transcription genes involved in innate immunity. This decrease in nuclear NF-κB could, as the authors suggest, result in attenuated responses in certain aspects of innate immunity.

Interventions that target psychosocial stressors have been implemented among diseased populations in order to counterbalance the effects of stress on innate immune functioning. Yoga as a form of complementary medicine has increasingly been used by breast cancer survivors in attempts to provide immunological benefits. Iyengar yoga intervention was used in this study as a means to provide relaxation and meditation techniques in order to examine its effectiveness in lowering psychosocial stressors and its effects on nuclear expression of NF-κB in lymphocytes. As reported, there was no significant yoga effect on psychosocial parameters among the breast cancer survivors. This result may indicate that psychosocially, this group of cancer survivors was a very stable population, and therefore there was no yoga effect to report.

Of greater interest, may be the relationships between stress and nuclear NF-κB expression and stimulation index. The subscales that correlated most strongly with NF-κB stimulation index of NF-κB in unstimulated lymphocytes were 1) the personal
meaning hardships associated with breast cancer and treatment; 2) the hardships
associated with monitoring treatment and disease-related symptoms; 3) the global
perception of current hardships imposed by illness and treatment; 4) the global perception
of illness and treatment hardships since the time of original diagnosis and 5) overall
scores on the DOII.

A major finding of this investigation was that an increase in perceived stress was
correlated with an increase in nuclear NF-κB in unstimulated lymphocytes and also to a
decrease in lymphocyte stimulation index of NF-κB to rIL-2 and PHA/PMA. A lower
N:C ratio of NF-κB in unstimulated lymphocytes was also observed among these breast
cancer patients with increased stress, indicated by increased DOII scores. These findings
suggest that psychosocial stress may indeed be related to NF-κB nuclear expression and
NF-κB response to stimulation in lymphocytes of breast cancer survivors. The effects of
increased psychosocial stressors may result in an increased nuclear NF-κB expression
and therefore a decreased NF-κB stimulation index in lymphocytes of breast cancer
survivors.
CONCLUSION

It was not possible to determine if women with stage II-IV breast cancer who participated in an 8-week yoga intervention had altered nuclear expression of NF-κB in lymphocytes stimulated with rIL-2 and PHA/PMA. The degree of stimulation was, in part, dependent upon the expression of nuclear NF-κB in unstimulated cells. There appears to be a relationship between NF-κB and perceived psychosocial stress, with increased stress levels being correlated with increased nuclear NF-κB expression and a decreased rIL-2 and PHA/PMA stimulation index of NF-κB in lymphocytes of breast cancer survivors. The biological relevance of these results requires further investigation; however, these findings lend support to the hypothesis that NF-κB regulation and illness-related stressors are inter-related. Further explication of the complex interplay of both types of variables could benefit breast cancer survivors.
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4. Sigma Xi Graduate Student Research Award
5. AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: Human rIL-2 from Dr. Maurice Gately, Hoffmann-La Roche Inc.
REFERENCES


APPENDIX B
List of Tables

Table 1. Characteristics of Breast Cancer Survivors

Table 2. DOII Scores of Breast Cancers Survivors
Table 1. Characteristics of Breast Cancer Survivors

<table>
<thead>
<tr>
<th></th>
<th>Yoga Mean</th>
<th>Yoga SD</th>
<th>Control Mean</th>
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<td>IV</td>
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Table 2. DDI Scores of Breast Cancer Survivors

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<td>Monitoring symptoms</td>
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<tr>
<td>Accommodating to treatment</td>
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<tr>
<td>Relationship with provider</td>
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<td>0.06</td>
</tr>
<tr>
<td>Information exchange about treatment</td>
<td>3</td>
<td>0.20</td>
</tr>
<tr>
<td>Treatment</td>
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<td>0.45</td>
</tr>
<tr>
<td>Global hardships perceived now</td>
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<td>5.33</td>
</tr>
<tr>
<td>Global hardships since diagnosis</td>
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<td>6.00</td>
</tr>
<tr>
<td>Overall scores</td>
<td>3</td>
<td>0.60</td>
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List of Figures

Figure 1A. Stimulation index of rIL-2 responders for yoga and TAU pre (T₁) - and post (T₂) - intervention. Values represent mean ± SEM. Significant group difference at T₂ (p = 0.015).

Figure 1B. Stimulation index of PHA/PMA responders for yoga and TAU at T₁ and T₂. Values represent mean ± SEM. Significant group effect at T₂ (p = 0.0495).

Figure 2A. Nuclear NF-κB in rIL-2 Responders. rIL-2 stimulation index of nuclear NF-κB from unstimulated lymphocytes at T₁ and T₂. The relative amount of NF-κB present before stimulation is expressed as luminescence count per second (lcps). R² = 0.817, p<0.001.

Figure 2B. Nuclear NF-κB in PHA/PMA Responders. PHA/PMA stimulation index of nuclear NF-κB from unstimulated lymphocytes at T₁ and T₂. The relative amount of NF-κB present before stimulation is expressed as luminescence count per second (lcps). R² = 0.455, p=0.016.

Figure 3A. N:C of NF-κB in rIL-2 Responders. Stimulation index is associated with NF-κB nuclear:cytosolic (N:C) ratio in unstimulated lymphocytes. R² = 0.427, p=0.029.

Figure 3B. N:C of NF-κB in PHA/PMA Responders. Stimulation index is associated with NF-κB nuclear:cytosolic ratio in unstimulated lymphocytes. R² = 0.388, p=0.031.

Figure 4. Association between baseline nuclear NF-κB nuclear expression and baseline N:C of NF-κB. R² = 0.3215, p=0.009.

Figure 5. Association between rIL-2 stimulation index of NF-κB and PHA/PMA stimulation index of NF-κB. R² = 0.5699, p < 0.001.

Figure 6. Hardships associated with breast cancer and treatment related to personal meaning for yoga intervention and TAU at T₁ and T₂. Values represent mean ± SEM. Significant group difference (p=0.002) at T₂.

Figure 7. Hardships imposed by breast cancer treatment related to social relationships for yoga intervention and TAU at T₁ and T₂. Values represent mean ± SEM. Significant group difference (p=0.039) at T₂.

Figure 8. Association between the hardships associated with breast cancer and treatment related to personal meaning and N:C ratio of NF-κB in unstimulated lymphocytes at T₁. R² = 0.4134, p=0.045.

Figure 9. Association between the overall hardships associated with breast cancer and PMA/PHA stimulation index of NF-κB in lymphocytes at T₁. R² = 0.4232, p=0.042.
Figure 10. Association between the hardships associated with breast cancer and monitoring treatment and disease-related symptoms and stimulation index of NF-κB in lymphocytes of responders to PMA/PHA stimulation at $T_1$. $R^2 = 0.772$, $p=0.032$. 
Figure 1A

![Graph showing stimulation index index (%)](image)

- T1
- T2

Legend:
- Yoga
- TAU

* Significant difference
Figure 1B

![Graph showing stimulation index (%) for T1 and T2 with corresponding bars for Yoga and TAU groups.](image)
Figure 6
Figure 7
Figure 8

baseline N: C luminescence units (lcp)

personal meaning (Doll, 0-4)

GROUP
- Chem
- TAU
- Yoga

Total Population

Rsq = 0.4139
Figure 10

[Graph showing monitoring symptoms (DOII, 0-4) vs. PHA/PMA stimulation index (%).]

GROUP:
- Chemo
- TAU
- Yoga

Total Population

Rsq = 0.7222
IYENGAR YOGA CLASSES FOR WOMEN WITH BREAST CANCER

I. Week 1-8
   a. Day 1: Introduction, class organization, class schedule
      i. Introduction to Seated Meditation, Yogic Philosophy
      ii. Introduction to Asanas selected standing and seated Asanas, Savasana
   b. Day 2:
      i. Meditation, Yogic Philosophy
      ii. Seated Asanas at wall, Mt. Pose at wall, Variations of D.Dog, beginning back bends, Variations of twists, Savasana
   c. Day 3:
      i. Meditation, Yogic Philosophy
      ii. Variations of Mt. Pose, Supine twists, Variations of supine poses, beginning back bends, intro to triangle, beginning supine inversion, Savasana
   d. Day 4:
      i. Meditation, Yogic Philosophy
      ii. Variations of seated poses, Variations of Mt. pose, Triangle at wall, seated twists, Variations of supine hip openers at wall, beginning supine inversion, Savasana
   e. Day 5:
      i. Meditation, Yogic Philosophy
      ii. D. Dog away from wall, Standing fwd bend, beginning standing and seated hip openers, triangle at wall, supine inversion, Savasana
   f. Day 6:
      i. Meditation, Yogic Philosophy
      ii. Supine hip openers, Mt. pose, leg strengtheners, forward bend standing, Warrior II, supine inversion, Savasana
   g. Day 7
      i. Meditation, Yogic Philosophy
      ii. Chest openers, seated hip openers, modified sun salutation, leg strengtheners, Warrior II, side stretches, modified standing poses, supine inversion, Savasana
   h. Day 8
      i. Meditation, Yogic Philosophy
      ii. Seated hip openers, supine hip openers, Triangle at wall, Warrior II, Modified standing poses, partner D. Dog, Savasana
   i. Day 9
      i. Meditation, Yogic Philosophy
      ii. Intro to complete modified Sun Salutation, seated hip openers, shoulder openers, supine twists, Savasana
j. Day 10
   i. Meditation, Yogic Philosophy
   ii. Supine stretches, sun salutations, hip openers at wall, Tree at wall,
       standing inversion/hip opener, supine hip openers, Tree away from
       wall, shoulder openers, supine bridge, Savasana

k. Day 11
   i. Meditation, Yogic Philosophy
   ii. Sun Salutations, standing poses, Warrior I modified, modified
       supine back bends, Savasana

l. Day 12
   i. Meditation, Yogic Philosophy
   ii. Seated hip openers, Triangle away from wall, modified standing
       poses, Warrior II, Leg strengthening, standing poses at wall, supine
       modified back bends, supine inversion, savasana

m. Day 13
   i. Meditation, Yogic Philosophy
   ii. Sun Salutations, hip openers seated, D. Dog, modified standing
       poses at wall, Savasana

n. Day 14
   i. Meditation, Yogic Philosophy
   ii. Supine leg stretches, hip openers, D. Dog, plank at wall, restorative
       poses, Savasana

o. Day 15
   i. Meditation, Yogic Philosophy
   ii. Tree at wall & away from wall, standing poses, D.Dog variations,
       supine modified back bends, intro to Up.Dog, Sun Salutations,
       Savasana

p. Day 16
   i. Meditation, Yogic Philosophy
   ii. Seated hip openers, D. Dog variations, plank at wall, Triangle,
       Warrior II, Standing fwd bend, partner D. Dog, Tree with partners,
       supine restorative, Savasana