Soy Isoflavone Supplementation Does Not Alter Distribution of Circulating Lymphocytes or Natural Killer Cell Activity in Postmenopausal Women

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ABSTRACT

A growing body of evidence has demonstrated that soy isoflavone consumption may protect against the development of various chronic diseases. This defense could be linked to isoflavone-induced alterations in immune function. However, to date, no study has examined the effect of soy isoflavone supplementation on human immunity *in vivo*. Establishing whether isoflavones affect immunity in aging adults is particularly relevant since compromised immune function has been observed in this population. Therefore, the purpose of this double-blind, placebo-controlled, 4-wk intervention trial was to investigate whether supplementation with soy isoflavones influenced the distribution and/or function of specific lymphocytes in postmenopausal women. Healthy postmenopausal women (50-69 y), who were not using hormone replacement therapy, were randomly divided into 2 treatment groups. The experimental group (n=9) consumed two-50 mg soy isoflavone tablets/d for 4 wk, while the control group (n=9) received placebo tablets. Fasting blood samples were drawn at baseline and on d 28 to assess distribution of T-helper cells ($CD3^+CD4^+$), T-cytotoxic cells ($CD3^+CD8^+$), total T lymphocytes ($CD3^+$), B lymphocytes ($CD19^+$) and natural killer (NK) cells ($CD16^+CD56^+$) via flow cytometry. Cytotoxicity of NK cells was quantified based on lactate dehydrogenase release of lysed K562 cancer cells following co-culture with NK cells from subjects. Analysis of plasma isoflavone concentrations by HPLC demonstrated a significant increase ($p<0.005$) in plasma genistein concentration in the experimental group after 4 wk of supplementation. However, there was no alteration in lymphocyte distribution or NK cell activity in response to isoflavone supplementation, suggesting that short-term soy isoflavone supplementation does not alter these parameters of immunity in healthy postmenopausal women.

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Chapter 1: Introduction

In recent years, researchers have tried to unravel the highly complex relationship between nutrition, immunology and cancer. It is well known that dietary antioxidants can neutralize the damaging effects of reactive oxygen species produced by normal metabolism, exercise and certain immune cells (1). However, other possible links between diet, immune function and tumor growth are still poorly understood. For example, although specific molecular mechanisms remain unclear, certain nutrients and other dietary components seem to be directly involved in regulating gene expression (2) and cell-cell communication between non-immune, immune and tumor cells (3). Clearly, additional research is warranted to further identify the role of dietary components in modulation of immune parameters and tumor development.

According to the National Cancer Institute, breast cancer accounts for 39% of all cancers diagnosed in women and it is the leading cause of death for women aged 15-54 y (4). Age-adjusted breast cancer rates reveal that older women are particularly vulnerable. While breast cancer incidence and mortality rates for women under 65 y are 74.5 and 14.7 per 100,000, respectively, the corresponding rates for women 65 y and older are 440.6 and 122.9 per 100,000 (4). Yet among Asians the risk for cancers is significantly lower, particularly for hormone-dependent tumors such as breast cancer. In fact, compared to the United States and Western Europe, age-adjusted mortality rates from breast cancer are 2- to 8-fold lower in Asian societies (5). However, Asian women who move to the United States and subsequently adopt a Western diet become as susceptible to breast cancer as their American counterparts within one to two generations (6,7). In addition, substantial increases in breast cancer rates have paralleled the embracing of Western diets among Asians living in affluent urban areas of Japan, China and Singapore (8). These epidemiological trends suggest that differences in breast cancer rates among American and Asian women are due, in part, to differences in their diets.

One major dietary difference is the per capita consumption of soy, which is estimated to be 20-50 times greater among Asians compared to Americans (5). Soybeans are rich sources of the isoflavones genistein and daidzein, which continue to be investigated for their potential in reducing the risk for cardiovascular diseases, osteoporosis, certain types of cancer, and alleviating symptoms of menopause (6). The fact that Asians have significantly lower breast

cancer mortality rates compared to Americans seems to suggest that soy isoflavones may also contribute to reducing the risk of tumor initiation and/or progression.

However, proposed mechanisms of *how* soy may reduce the risk of certain cancers remain highly speculative. Soy isoflavones may affect human *immune functions* as weak estrogen agonists and/or antagonists. Both genistein and daidzein possess weak estrogenic activity due to their structural similarity to 17β-estradiol (9). Since the human immune system is, in part, regulated by steroid hormones (10), soy isoflavones may influence immunocompetence via estrogen-receptor mediated mechanisms. In addition, soy isoflavones possess a variety of immune characteristics, including antioxidant, antiproliferative, anti-inflammatory and differentiation-inducing properties. Data from recent *in vitro* and animal studies have indicated that isoflavones may either enhance or suppress immunocompetence, depending on their concentration, the target tissue and a number of other factors.

In October of 1999, the US Food and Drug Administration (FDA) authorized a new health claim stating that 25 g/d of soy protein, as part of a healthy diet, may reduce the risk of heart disease (11). Soy food products and soy tablets may already be widely consumed by postmenopausal women for the prevention of heart disease and osteoporosis as well as the alleviation of menopausal symptoms. In fact, according to the 2001 Soy Food Guide, soy food sales in this country have increased from $ 2 million to just over $ 2 billion in the last 20 years (12). Yet, despite the growing popularity of soy foods and supplements, no study has investigated the effects of soy isoflavone consumption on immune parameters in *humans*. Therefore, this study was designed to examine the relationship between short-term soy isoflavone supplementation and specific aspects of immunity in healthy postmenopausal women.

Chapter 2: Literature Review

Human Immune System

Background

The human immune system can be divided into two main branches, namely the innate system, which is nonspecific or antigen-independent, and the acquired immune system, which elicits specific, antigen-dependent responses (10). The acquired immune system is further subdivided into humoral immunity and cell-mediated immunity. While humoral immunity is dependent on B lymphocytes (B cells) which originate from bone marrow stem cells and are stored in the spleen, cell-mediated immunity is dependent on T lymphocytes (T cells) which also originate from bone marrow stem cells, but mature and are stored in the thymus. Upon exposure to a foreign material, or antigen, activated B cells produce antibodies, or immunoglobulins, that bind specifically to the corresponding antigen. The antibody-antigen complex can then be effectively eliminated via complement-activated phagocytic macrophages (10).

Instead of secreting antibodies, the three major types of T cells can bind directly to antigen presenting cells (APC) (13). The two types of T-helper (TH) cells, called T helper-1 (TH-1) and T helper-2 (TH-2) cells, assist macrophages and B cells, respectively. The third type of T lymphocytes, called T-cytotoxic cells, is capable of destroying cancer cells and cells infected by intracellular pathogens (14). While TH cells express CD4 molecules, T-cytotoxic cells express CD8 molecules on the cell surface. In addition, all three types of T cells express the CD3 molecule, also called the T cell receptor (TCR) (14). To become activated T cells must bind to the APC via the TCR and either the CD4 or the CD8 cell surface receptors (13).

Natural killer (NK) cells are capable of killing tumor cells and virally-infected cells without first having to be activated by a membrane recognition system (15). NK cells, which make up about 15% of circulating lymphocytes, can also be distinguished by their unique cell surface markers, CD16 and CD56. Resting NK cells also express a portion of certain cytokine receptors such as the interleukin-2 (IL-2) receptor (15). In turn, cytokines represent a group of molecules which are produced by T cells, B cells and/or macrophages to serve as mediators between the different types of immune cells. For example, binding with IL-2 leads to stimulation

and subsequent activation of NK cells and T cells (16). Together, immune cells play an essential role in recognizing and eliminating tumor cells via a highly coordinated immune response (3). Yet, as part of the natural aging process, proliferation of T lymphocytes, B lymphocytes and NK cells appears to be inhibited (17). Thus, the fact that older aged women are disproportionately afflicted with breast cancer could be linked to the progressive aging of the immune system.

Human Immune System and Sex Steroid Hormones

Background

Studies have indicated that the immune response in females is generally greater than in males (10). For example, in Swiss Albino mice the immune response to bovine serum albumin (BSA) was stronger and longer in females compared to males (18). Similarly, female inbred mice reject skin allografts faster and more consistently than male inbred mice (19) and in humans, more females than males suffer from autoimmune diseases such as rheumatoid arthritis and lupus (10). A growing body of evidence suggests that these differences in male and female immune responses could be linked to the differences in sex steroid hormones and their impact on immune cell function.

In the case of humoral immunity, gonadal steroids could be linked to gender differences in antibody responses. Estradiol has been shown to inhibit suppressor T cells. This inhibition leads to accelerated B cell maturation followed by increased antibody synthesis (20). On the other hand, cell-mediated immunity seems to be suppressed by sex steroid hormones in general and estradiol in particular. According to the results of an *in vitro* study, the synthetic estrogen, fosfestrol (1 nM-1 µM), suppressed NK cell cytolysis of K562 tumor cells in a dose-dependent manner (21). Futhermore, animal studies have shown that gonadectomy alters the onset and progression of cancer. In hamsters this procedure decreased the number of mammary tumors, yet when these gonadectomized rodents were given a weekly dose of 0.05 mg estradiol, the number of tumors increased in both genders (22). This suggests that estradiol may inhibit the proliferation and/or activity of immune cells known to play a critical role in the defense against carcinogenesis.

In human prostate and breast cancer patients, estrogens have been shown to decrease T lymphocyte activation, thereby suppressing immune responses against these tumors (23,24). Furthermore, since human estrogens stimulate cell proliferation in hormone-sensitive tissues, these steroid hormones have also been associated with the progression of certain chemically-induced cancers (10). In sum, human sex steroid hormones seem to be partly responsible for the stronger antibody responses typically seen in females and for a general decrease in T lymphocyte proliferation, which in turn could lead to suppressed cancer inhibition responses. It is unknown whether soy isoflavones, which can reach much higher plasma concentrations than endogenous estrogens, would elicit similar responses due to their ability to exert weak estrogenic activity.

Interestingly, the results of a recent Japanese study have shown an inverse correlation between dietary intakes of soy products and serum estradiol concentrations in premenopausal women (25). Hence, one of the mechanisms by which soy isoflavones may indirectly alter human immune function may be by lowering circulating concentrations of estradiol. Based on the literature, such isoflavone-induced changes in estradiol metabolism could result in enhanced humoral immunity and/or suppressed cell-mediated immunity.

Soy Isoflavones

Background

Flavonoids (phenylbenzo-γ-pyrones) are heterocyclic phenols which naturally occur in plants. Based on the position of their phenyl group, these phytochemicals can be subdivided into two major families, the flavones and isoflavones (26). The phenyl group is also considered to be a prerequisite for estrogenic activity (27). In fact, the chemical structure of flavonoids is so similar to 17β-estradiol (Figure 1) that these plant compounds can bind to estrogen receptors expressed on mammalian cells. For this reason flavonoids are also referred to as *phytoestrogens*, which may act as weak estrogen agonists and/or estrogen antagonists with a potency of 10^{-2} to 10^{-3}-fold the activity of 17β-estradiol and estrone (9). In Japanese adults consuming soy-rich diets typical isoflavone plasma concentrations of 3-5 μM (28) are approximately 1,000 times higher than peak plasma concentrations of endogenous estrogens in premenopausal women (29).

Thus, at such relatively high concentrations, soy isoflavones may exert powerful effects even as weak estrogenic compounds.

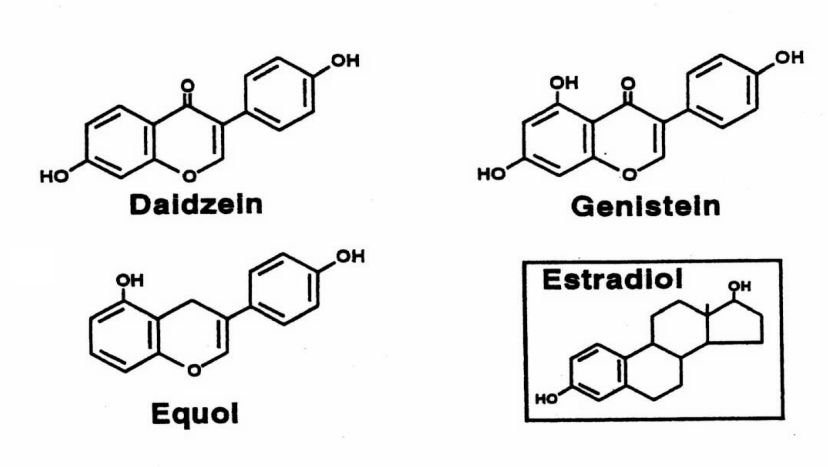


Figure 1: Principal isoflavone aglycones of soy (daidzein and genistein), the bacterial product of isoflavone metabolism (equol), and the female sex steroid hormone (estradiol) (30).

While flavones are found in a wide range of plant foods, natural sources of isoflavones are limited to soybeans, chickpeas, a few other legumes, clovers and bluegrass (31). However, due to the abundance of processed soybean products such as soy flour, tempeh, tofu, miso, soy milk, soy noodles, soy hot dogs, tofu yogurt and textured vegetable protein, dietary sources of soybeans, and hence isoflavones, are quite diverse. Depending on the type of soybean, the degree of processing, and the amount of added ingredients, the total isoflavone content of these soy products varies from as little as 8.5 mg/100 g in dry soy noodles to as much as >300 mg/100 g in soy flours (32).

Despite the abundance of soybean products, the average American and Western European consumes less than 5 mg of isoflavones a day (33). Asians, on the other hand, typically consume 50-100 mg/d (33,34) and in Japan the per capita consumption of isoflavones is estimated to be as high as 200 mg/d (32). More recently, soy isoflavone tablets and powders have become increasingly available in American grocery stores, pharmacies and natural food stores. These dietary supplements have gained enormous popularity for their potential benefits in cardiovascular, bone and prostate health, as well as the relief of menopausal symptoms (12).

Future use of soy products is expected to increase even more dramatically in response to a recent FDA ruling. According to this new health claim, 25 g/d of soy protein may reduce the risk of heart disease (11). Since soy protein isolates contain 103-145 mg of total isoflavones per 100 g, this recommended amount of soy protein would provide approximately 26-36 mg of soy isoflavones (32).

In nature, isoflavones occur as β-glucoside conjugates (30). Genistin, the most abundant isoflavone, represents roughly two-thirds of the entire soybean isoflavone content. The remaining one-third is composed primarily of daidzin and very small amounts of glycitin (30). Once ingested the isoflavonoid β-glucosides, **genistin** and **daidzin**, are converted by intestinal bacteria into their respective aglycones, **genistein** and **daidzein** (35). Isoflavone bioavailability therefore depends in part on the rate and the extent of microbial hydrolysis. For example, only about 30% of the human population seems to have the appropriate flora for synthesizing equol, one of the major isoflavone metabolites (35,36). Studies have also shown that the bioavailability of the less concentrated isoflavone, daidzein, is generally greater than that of genistein (37,28). In addition, while fermentation of soy products enhances isoflavone bioavailability, it also reduces overall isoflavone content (38).

Once absorbed, isoflavonoid aglycones travel via the portal vein to the liver where they are readily conjugated almost exclusively with glucuronic acid and to a much smaller extent with sulfate (30). After hepatic conjugation, isoflavone metabolites enter the peripheral circulation. Even at pharmacological doses urinary isoflavone excretion is directly proportional to isoflavone intakes, indicating that soy isoflavones are readily absorbed and metabolized even at extremely high concentrations (39). After a soy-rich meal this excretion can increase up to 1,000-fold (35). In healthy adult males consuming 60 g baked soybean powder containing isoflavone glycones, the half-lives of genistein and daidzein in plasma have been reported to be 8.4 h and 5.8 h, respectively (40). With such transitory half-lives, *in vivo* isoflavone concentrations are likely to be pulsatile, corresponding to the timing and dosage of dietary soy intake. Thus, it is possible that isoflavone-induced effects on immune function are also pulsatile in nature.

Isoflavones as Modulators of Immune Cells

Weak Estrogen Agonists and/or Antagonists

Since the chemical structure of soy isoflavones is remarkably similar to that of sex steroid hormones, which have been shown to modulate immune cell responses, it has been postulated that these dietary components may exert similar effects on the human immune system. Genistein may suppress normal estrogen secretion and/or estrogen activity in estrogen-sensitive tissues by competing for estrogen receptor (ER) binding (41). According to Nagata et al. (25), soy consumption was inversely related to serum estradiol concentrations in 50 healthy premenopausal Japanese women after accounting for such confounding factors as age, BMI, menstrual cycle length, energy intakes, dietary fats and crude fibers. In another study, plasma estradiol levels of pre- and postmenopausal Caucasian American women were 30-70% and 200% higher, respectively, compared to the plasma estradiol levels of their age-matched Asian cohorts (29). Thus, soy-induced depression of circulating estradiol levels could be one of the mechanisms by which soy isoflavones exert their effects on human immunocompetence.

Inhibition of Tyrosine-Specific Protein Kinases

The potential of genistein to exert immunomodulatory effects could also be linked to its role as an inhibitor of tyrosine-specific protein kinases (PTK) (42,43). Tyrosine phosphorylation plays an integral part in cell proliferation. Since regulation of proliferation is an important component of normal immune function, genistein-stimulated PTK inhibition could affect various parameters of immunity. According to results obtained by Minoguchi et al. (44), when human TH-1 and TH-2 cells were incubated with genistein, inhibition of tyrosine phosphorylation led to altered cytokine production in both types of T cell clones. Furthermore, tyrosine phosphorylation seems to be involved in the activation of B lymphocytes and NK cells (45,46). In sum, these preliminary data suggest that genistein, as a PTK-inhibitor, may play a critical role in regulating immune function.

Induction of Cell-Cycle Arrest and/or Apoptosis

Isoflavone-induced inhibition of cell proliferation could be the result of the stimulatory effect of genistein on cell-cycle arrest and apoptosis. According to Spinozzi and associates (47), when Jurkat cells (T cell leukemia cell line) were treated with genistein, G_2/M-phase arrest and S-phase derangement occurred at 5-10 µg/ml and 20-30 µg/ml, respectively. In turn, these cell-cycle aberrations led to apoptotic death of the genistein-treated leukemia cells (47). More recently, a study conducted by Li and associates (48) found that genistein, at a concentration of 5 µM, inhibited cell growth in the MDA-MB-231 breast cancer cell line via regulation of apoptosis-related gene expression. Both of these *in vitro* studies provide evidence for the ability of genistein to modulate cell growth, which raises two important points. First, genistein may also be able to inhibit *in vivo* tumor cell growth via induction of cell-cycle arrest and/or apoptosis. Secondly, genistein may also be capable of regulating the growth of proliferating, non-tumor cells, such as immune cells, which could be detrimental to optimal functioning of the immune system. However, these data were generated from *in vitro* investigations using tumor cell lines. It is not known if genistein, at physiologically attainable concentrations, would also inhibit the proliferation of immune cells in humans by inducing cell-cycle arrest and/or apoptosis.

Induction of Cell Differentiation

Differentiated cells are far less susceptible to genetic mutations than undifferentiated cells. Thus, the apparent chemopreventive potential of soy could be linked to isoflavone-induced cell differentiation (49). In 1995, Lamartiniere and associates provided *in vivo* evidence in support of this hypothesis (50). Mammary glands of genistein-treated female rats had only a few immature terminal end buds and significantly more fully differentiated lobular structures than the mammary glands of rats which had not been treated with the soy isoflavone. Furthermore, the mean number of DMBA-induced tumors in the genistein-treated rats was only 3.67, compared to the mean number of 6.35 tumors in the control animals (50). While this genistein-induced cell differentiation in hormone-sensitive tissues may inhibit carcinogenesis, isoflavone-induced cell differentiation of *immune cells* could dramatically alter *immune function*. Recent *in vitro* data suggest that soybean isoflavones may indeed enhance the differentiation of human immune cells

in a time- and dose-dependent manner. Peak genistein-induced expression of maturation markers was measured on days 4 and 6 at 20 μg/ml and 10 μg/ml in HL-205 and K562-J cell lines (clones of human promyelocytic HL-60 and erythroid K562 leukemia cells), respectively (51). This *in vitro* evidence of genistein-induced immune cell differentiation could be linked to alterations in immune function, particularly if isoflavone exposure occurs during early development of the human immune system.

Modulation of Nonspecific Immunity

Since lymphocytes express estrogen receptors, phytoestrogens have the potential to modulate immune cell activity via ER-mediated pathways (10). Yet, to date clinical experiments on isoflavones as modulators of immune function are virtually nonexistent. Similarly, very few *in vitro* and animal studies have investigated this. In 1997, a study conducted by Zhang and colleagues (52) was one of the first to generate *in vivo* evidence for daidzein-induced nonspecific immune function. Swiss mice fed either 20 or 40 mg/kg daidzein for 7 d showed significant increases in thymus weights and phagocytic activity of peritoneal macrophages. Lower daidzein feedings of 10 mg/kg did not seem to be effective (52). Thus, in mice this dose-dependent immune response was observed only at isoflavone concentrations several times greater than those typically seen in Asians consuming a soy-rich diet.

Potentiation of Lymphocyte Activation

In 1997, Wang and associates examined the effects of genistein and daidzein on mitogen-induced activation of murine lymphocytes (53). Using daidzein at a range of 0.01-10.0 μM, responses of Concanavalin A (Con A)-induced and *Escherichia coli* lipopolysaccharide (LPS)-induced splenocytes were significantly enhanced by 122-149% and 111-127%, respectively. Genistein treatments, in contrast, did not significantly alter spleen cell proliferation in response to mitogen stimulation. In addition, only daidzein significantly induced interleukin-2 (IL-2) and IL-3 from Con A-stimulated splenocytes (53). The researchers concluded that potentiation of lymphocyte activation may actually not involve ER-dependent mechanisms since daidzein

caused potentiation while genistein did not, even though both isoflavone metabolites are known to have similar binding affinities for estrogen receptors in ER-sensitive tissues (53). This preliminary evidence suggests that the cellular mechanism(s) and the potency of soy isoflavones are sensitive to levels of circulating estrogens, amounts of dietary isoflavones, and, perhaps most importantly, the target tissue as well as the timing of exposure to isoflavones. Thus, additional research is needed to verify this potentiation of lymphocyte activation and to further investigate the mechanisms involved.

Zava and Duwe (54) investigated the effects of genistein on cell growth and on estrogen-regulated pS2 induction in various human breast cancer cell lines. Although genistein effectively inhibited the growth of ER+ and ER- breast cancer cell lines at high concentrations of $\geq$10 µM, at lower, physiologically relevant concentrations of 1 nM –10 µM genistein seemed to have the opposite effect. Genistein-induced cell proliferation peaked between 0.1-1.0 µM, equaling the stimulatory effect of estradiol at 1 nM (54). However, the influence of these concentrations of genistein on human *immune cells* remains unknown. Therefore, it is logical to explore this relationship, since soy phytoestrogens clearly affect estrogen metabolism and, as noted earlier, estradiol appears to influence several parameters of immune function.

Activation of Human Natural Killer Cells

NK cells play a critical role in halting the progression of tumors and certain infections. In a recent *in vitro* experiment conducted by Zhang and colleagues (55), genistein significantly stimulated NK cell-induced K562 cancer cell killing at a concentration range of 0.1-0.5 µM. However, at higher concentrations (5-50 µM) genistein significantly inhibited NK cell-mediated cytotoxicity (55). Thus, at pharmacological doses, genistein may actually exert immunosuppressive effects in humans. This isoflavone-induced inhibition of NK cell function may, in part, be explained by the role of genistein as a specific PTK inhibitor, since the activation of NK cell cytotoxicity is dependent on tyrosine phosphorylation (46). As mentioned previously, Japanese adults consuming soy-rich diets typically have isoflavone plasma concentrations of 3-5 µM (28). Therefore, based on this *in vitro* evidence, dietary soy intake is more likely to enhance, rather than inhibit, the potentiation of human peripheral NK cells (55).

Hence, the possibility that physiologically relevant concentrations of soy isoflavones may enhance immune function via the potentiation of NK cells warrants further investigation.

It is also possible that genistein may actually exert an effect directly on K562 cancer cells, either instead of or in addition to modulating the activity of NK cells. In fact, Baral et al. (56) examined the effects of the antiestrogen, tamoxifen, and estradiol at various concentrations (1 nM – 1 μM) on YAC-1 murine lymphoma cell killing induced by splenic NK cells in female Fisher and Wistar-Furth rats. It was found that tamoxifen and estradiol enhanced target cell cytolysis at pharmacologic as well as physiologic concentrations (56). However, the estradiol-mediated increase in sensitivity of target cells to lysis was negated by the simultaneous decrease in NK cell-induced target cell killing in the presence of this hormone (56). The authors proposed that the observed target cell sensitization to lysis may involve priming of the cell for apoptosis. Since both tamoxifen and estradiol enhanced target cell susceptibility to NK cell lysis, it seems plausible that the phytoestrogen genistein may also enhance target cell destruction by having a similar effect on these cells.

Potential Immunosuppressive Mechanisms

In addition to the aforementioned inhibition of NK cell activity in the presence of high concentrations of genistein, recent evidence suggests that, in mice, this isoflavone may also suppress immune function via thymic atrophy and alterations in lymphocyte subpopulations (57). In ovariectomized female mice, injection of genistein (5 mg/d) for 21 d resulted in an 80% reduction of thymic weight, while injection of this isoflavone at a lower dose (2 mg/d) for 7 d led to a 70% decrease in thymic weight (57). In addition, the number of $CD4^+$ and $CD4^+CD8^+$ T cells decreased in genistein treated mice compared to the control animals (57). Results of another recent animal study suggest that genistein may hold potential as an immunosuppressive agent in recipients of organ transplants. When Lewis rats were fed soy isoflavone-enriched protein (containing 5 mg/g isoflavones) beginning 2 wk prior to cardiac transplantation, allograft rejection was significantly delayed compared to the control animals receiving casein as a protein source (58).

The two former studies suggest that in the mouse and rat model, high concentrations of genistein may actually suppress the immune response. However, since isoflavones seem to exert a biphasic effect on immune cells depending on their concentrations, it is not clear how soy isoflavone supplementation at physiologically-attainable doses may influence indices of immunity. Furthermore, the nature and/or extent of isoflavone-induced modulation of immune function may be quite different in human subjects than in the animal models.

In short, a growing body of evidence suggests that nutrition, immune function and carcinogenesis are closely connected. Soybeans are rich sources of the isoflavones, genistein and daidzein, which are considered to be phytoestrogens due to their ability to exert weak estrogenic activity. Since steroid hormones in part regulate the human immune system, phytoestrogens may alter human immunity by acting either as estrogen agonists or antagonists. In addition, soy isoflavones may modulate immune functions via ER-independent properties, such as their antioxidant, anti-inflammatory, antiproliferative, and differentiation-inducing abilities. Results from recent *in vitro* and animal studies suggests that high doses of genistein may actually suppress immunocompetence, as indicated by decreased NK cell activity in humans, thymic atrophy and altered lymphocyte distributions in mice, and delayed rejection of cardiac allografts in rats. Furthermore, both genistein and daidzein work in a dose-dependent manner, particularly as inhibitors of cell proliferation. Thus, their physiological impact, especially as phytoestrogens, may vary not only between males and females, but also between pre- and postmenopausal women. Establishing whether isoflavones affect immunity in aging adults is particularly relevant since compromised immune function has been observed in this population. Yet, to date no study has been conducted to measure the effect of soy isoflavones on parameters of human immune function.

Based on the reviewed findings, the potential for soy isoflavones to modulate the human immune system warrants more in depth exploration. Thus, the objective of this investigation was to determine the effects of soy isoflavone consumption on the distribution of circulating lymphocytes and the activity of NK cells in postmenopausal women. To our knowledge, this study was the first *in vivo* study to examine the effects of soy supplementation on cell-mediated immunity in this population sector.

Chapter 3: Manuscript

Soy Isoflavone Supplementation Does Not Alter Immune Parameters in Postmenopausal Women

Abstract

A growing body of evidence has demonstrated that soy isoflavone consumption may protect against the development of various chronic diseases. This protection could be linked to isoflavone-induced alterations in immune function. However, recent *in vitro* and animal studies suggest that soy isoflavones may either enhance or suppress immunocompetence, depending upon the isoflavone concentration, target tissue, and a number of other factors. To date, no study has investigated the effect of dietary soy isoflavone supplementation on immune parameters *in humans*. Establishing whether isoflavones affect immunity in aging adults is particularly relevant since compromised immune function has been observed in this population. Therefore, the purpose of this double-blind, placebo-controlled, 4 wk intervention trial was to investigate whether supplementation with soy isoflavones alters indices of immune function. Healthy postmenopausal women (50-69 y), who were not on hormone replacement therapy, were randomly divided into 2 treatment groups. The supplemented group (n=9) consumed soy isoflavone tablets (100 mg/d) for 4 wk, while the control group (n=9) received placebo tablets. Fasting blood samples were drawn at baseline and on d 28 to assess circulating lymphocyte distribution, lymphocyte proliferation, cytokine production, and NK cell cytotoxicity. In addition, plasma concentrations of genistein and daidzein were quantified at baseline and at the end of the intervention period. Despite high individual variability among subjects, there was a significant increase ($p<0.005$) in plasma genistein concentration in the supplemented group. However, all assessed immune parameters remained unchanged after supplementation and did not differ between the 2 treatment groups. In conclusion, this study suggests that short-term soy isoflavone supplementation at physiologically attainable concentrations does not alter the aforementioned immune parameters in healthy postmenopausal women.

Keywords: Isoflavones, genistein, daidzein, postmenopausal, lymphocytes, natural killer cells

Abbreviations: Con A, Concavalin A; IFNγ, interferon-γ; IL-2, interleukin-2; NK, natural killer; PBS, phosphate buffered saline; PTK, protein-tyrosine kinase.

Running Title: Isoflavones and Human Immune Function

Introduction

It is well documented that immune function declines in aging. For example, during aging both males and postmenopausal females show declines in anti-viral responses (1). Also, aging has been associated with reduced lymphocyte proliferation (2) and high levels of pro-inflammatory cytokines (3). In an effort to counteract the negative consequences of aging**,** nutritionists have investigated the effects of specific dietary components on various aspects of immunity. For example, vitamin E supplementation has been found to enhance cell-mediated immunity in healthy elderly populations (4). Continued investigation of the impact dietary constituents have on immune function, especially in aging individuals, is important.

Epidemiological data have suggested that consumption of soy foods, rich in the phytoestrogens genistein and daidzein, may have significant health benefits (5). Soy consumption has been associated with a decreased risk of cardiovascular disease (6), and may be beneficial in preventing the bone loss that leads to osteoporosis (7). Women also consume soy isoflavones to alleviate symptoms related to menopause (8). In addition to having weak estrogenic and antiestrogenic activities, isoflavones also possess characteristics such as antioxidant, antiproliferative, anti-inflammatory and differentiation-inducing abilities that may modulate immunity (9).

To date, limited studies have investigated the relationship between soy isoflavone consumption and immune function. However, data do exist suggesting isoflavones may either act to stimulate or suppress immunity depending on the dose and route of administration. In an *in vitro* study conducted by Wang et al. (10), daidzein at concentrations of 0.01-10 μM significantly increased lymphocyte proliferation as well as secretion of the cytokines IL-2 and IL-3 by murine splenocytes in response to Con A and lipopolysaccharide (LPS) stimulation. At similar concentrations, genistein alone did not have any immunostimulatory action (10). Since both isoflavones have similar estrogen receptor-binding affinities in estrogen responsive tissues (10), the findings of this study suggest that an estrogen-dependent mechanism might not be involved in the stimulatory activity of these isoflavones on lymphocyte proliferation and cytokine production. Furthermore, since a combination of genistein (1 μM) and daidzein (0.01 μM) had a

greater effect on murine splenocyte cultures than daidzein alone, more than one isoflavone-mediated mechanism might be involved (10). Another *in vitro* investigation showed that, genistein significantly stimulated natural killer (NK) cell-induced K562 cancer cell killing at a concentrations range of 0.1-0.5 μM (11). However, at higher doses (5-50 μM) genistein significantly inhibited NK cell-mediated cytotoxicity. Thus, genistein may act as a potential immunosuppressive agent at pharmacological concentrations. Since activation of NK cells is in part dependent on tyrosine phosphorylation (12), genistein, a known protein tyrosine kinase (PTK) inhibitor (13), may suppress NK cell activity via this mechanism. In addition, a recent study investigating the immunosuppressive action of isoflavones reported a decrease in total number of thymocytes as well as proportions of $CD4^+$ and $CD4^+$ $CD8^+$ cells in genistein-injected (5mg/d) ovariectomized female mice (14). Data from O'Connor and colleagues (15) demonstrated that administration of a diet containing high doses of soy isoflavones (5mg/g isoflavones) may act as an immunosuppressive agent to delay rejection of rat cardiac allografts. These conflicting observations regarding the immunomodulatory effect of soy isoflavones may be due to the fact that the mechanistic actions of isoflavones are highly sensitive to the amount, duration and route of administration of isoflavones.

Further justifying the need for determining the effect of isoflavone supplementation on immune function is a recent report of the synthetic isoflavone derivative, ipriflavone (16). In this study, a significant number of postmenopausal women consuming 600 mg ipriflavone/d (the equivalent of 60 mg isoflavones/d) for 12 mo were observed to have lymphocytopenia within 6 mo of treatment. This decrease in average lymphocyte numbers returned to normal within 24 mo in 81% of the patients, while the remaining 19% were followed up until the counts returned to normal. Thus, results from this trial further emphasize the need to clarify the role between isoflavone supplementation and immune function.

In October 1999, the US Food and Drug Administration authorized a new health claim suggesting that 25 g/d of soy protein can reduce coronary heart disease (17). Soy products and soy tablets may already be widely consumed by postmenopausal women for the prevention of some cancers, heart disease and osteoporosis as well as alleviation of menopausal symptoms. In fact, according to the 2001 Soy Food Guide, soy food sales in the United States have increased

dramatically from $2 million to just over $2 billion during the past two decades (18). Thus, it is important to clarify the role of soy consumption with respect to immune function in this population. However, virtually no human data on the relationship between soy consumption and immune response are available. Therefore, the objective of this *in vivo* study was to determine the effects of dietary soy isoflavone supplementation on lymphocyte distribution and proliferation, cytokine production and NK cell cytotoxicity in healthy postmenopausal women.

Materials and Methods

Subjects

Postmenopausal women aged 50-69 y were recruited to participate in this study. Exclusion criteria for selection included unusual dietary habits, regular consumption of soy-rich foods; high alcohol consumption; last menstrual cycle less than two years ago; history of disease; estrogen replacement therapy; use of any tobacco products and use of drugs, dietary- and/or herbal supplementation known to influence immune function. All subjects were free-living and maintained their normal dietary habits and physical activities. Approval for the use of human subjects was granted from the Virginia Tech Institutional Review Board and all subjects provided informed consent.

Experimental Design

In this double-blind, placebo-controlled intervention trial, all subjects were randomly assigned to one of two treatment groups. The experimental group (n=9) consumed two-50 mg soy isoflavone tablets/d (NovaSoy, Archer Daniels Midland, Decatur, IL) for 28 d, while the control group (n=9) received similar looking placebo tablets. The isoflavone tablets provided a total of 55-60 mg/d of isoflavone aglycones, which is representative of the amount typically consumed in Asian societies (19,20). To monitor compliance, subjects initialed a calendar each day following ingestion of the tablets and they were contacted by phone during the study. In addition, all participants completed 3-day dietary intake records twice during the intervention period to confirm usual dietary habits.

Fasting blood samples (24 ml) were collected from each subject on d 1 (pre) and d 28 (post) of the trial and used for determination of plasma isoflavone concentration, distribution of circulating lymphocytes, lymphocyte proliferation in response to mitogen, cytokine production by stimulated lymphocytes and NK cell cytotoxicity.

Determination of Isoflavone Concentration in Plasma

High Performance Liquid Chromatography (HPLC) was used for the determination of the soy isoflavones, daidzein and genistein in plasma according to the method of Xu et al. (21). Plasma (500 µl) was diluted in 10 ml of 80% methanol and centrifuged at 3000 x G for 10 min at 10^{o}C. After the methanol supernatant was poured off, the plasma residue was resuspended two additional times in 5 ml methanol and vortexed and centrifuged as above. The combined methanol extracts were evaporated and then dissolved in 5 ml of 1 M sodium acetate. Glucuronidase/sulfatase (50 µl H2 Type, Sigma G0876) was added and samples were incubated overnight at 37^{o}C. The isoflavones from this prepared hydrolysate were extracted on a C18 SPE reverse phase column (Fisher Scientific, Pittsburgh, PA) and then separated on a C18 250 x 4.6 mm column (Luna, Phenomenex, Torrance, CA), with 15% HPLC-grade methanol in HPLC grade water as the mobile phase. All samples were run in duplicates and fluorescein prepared in 80 % methanol was used as the internal standard. A standard curve was prepared using a series of genistein standards (200, 500, 1000 and 1200 ng) dissolved in 80% HPLC methanol and assayed to quantify the isoflavones.

Determination of Lymphocyte Distribution

Fluorescein isothiocyanate (FITC)-labeled mouse anti-human CD4 (clone SK3, T-helper cells), FITC-labeled mouse anti-human CD3 (clone SK7, pan-T cells), phycoerythrin (PE)-labeled mouse anti-human CD8 (clone SK1, T-cytotoxic cells), PE-labeled mouse anti-human CD19 (clone 4G7, pan-B cells) and PE-labeled mouse anti-human CD16+CD56 (clones B73.1 and MY31, NK cells) monoclonal antibodies (mAb) were used to label lymphocytes in 100 µl whole lysed blood (Becton Dickinson, San Jose, CA). Cells were double-stained with CD4 and CD8, CD3 and CD19, and CD3 and CD16+CD56 mAbs in duplicate. CD-Check Plus (Becton Dickinson) was used as a control. Cells were resuspended in 0.5 ml of 1.0% paraformaldehyde and stored at 4^{o}C until analysis the next morning. A total of 10,000 cells/sample was analyzed using an Epics XL flow cytometer (coulter Electronics, Hialeah, FL) at the Virginia Tech Flow Cytometry Lab. Lymphocyte populations were assessed by forward and side light scatter and subsequent analyses were made by gating this population. Results generated from this analysis were expressed as percentages of gated lymphocytes staining positively for each antibody.

Lymphocyte Isolation

Peripheral blood mononuclear cells were isolated using the double density Ficoll separation method (22). Heparinized blood (16 ml) from each subject was drawn into Vacutainer BD tubes containing Ficoll Paque reagent (BD Pharmingen, San Diego, CA). After centrifugation for 30 min, the lymphocytes were removed from the interface, washed twice with sterile PBS and resuspended in complete media (RPMI supplemented with 2 mM glutamine, 100 µg/ml Penicillin and 100 units/ml Streptomycin). An aliquot of the cell suspension was counted using a hemocytometer in the presence of Tryphan Blue dye and the isolated lymphocytes were used for proliferation, cytokine and natural killer cell cytotoxicity assays.

Lymphocyte Proliferation

The ability of isolated lymphocytes to proliferate in response to the T-cell mitogen, Concanavalin A (Con A) was measured using the ^{3}H-thymidine incorporation assay. Briefly, isolated lymphocytes from each subject were seeded into 96-well plates ($1x10^{6}$ cells per well) in complete media plus 10% autologous serum and incubated with 0, 2.5 or 5.0 µl/ml Con A . Following incubation at 37^{o}C and 5% CO_2 for 68 h, 20 µl of ^{3}H-thymidine (50 µCi/ml) were added to the cells and incubated for an additional 4 h at 37^{o}C. Cells were collected onto a glass fibre filter using a cell harvester (Skatron, Sterling, VA) and the incorporated radioactivity was measured using a liquid scintillation counter (Beckman LCS 6500). The results were obtained as counts per minute (cpm) and the effect of soy isoflavones on lymphocyte function was reported as the stimulation index calculated as the average counts per minute (cpm) of mitogen stimulated cultures minus the cpm of cultures without mitogen (baseline cpm) divided by the baseline cpm.

Analysis of IL-2, and IFN γ Protein Expression

Interleukin-2 (IL-2) and interferon-γ (IFNγ) production in response to Con A stimulation was measured in cell culture supernatant. Isolated lymphocytes ($7.5x10^{5}$ cells/well) were seeded into 24 well plates in complete medium plus 10% autologous serum containing 0 or 10 µg/ml Con A. Following incubation at 37^{o}C for 48 h, cell free supernatants were stored at –80^{o}C until

assayed. Enzyme linked immunosorbent assay (ELISA) analysis (OptEIA, PharMingen, San Diego, CA) was used to assess cytokine protein concentration in cell culture supernatant.

Natural Killer Cell-Mediated Cytotoxicity

NK cell-mediated cytotoxocity was determined according to the procedure of Korzeniewski and Callewaert (23) using the CytoTox 96 Kit (Promega Corporation, Madison, WI). K562 (target) cells (American Type Culture Collection, Manassas, VA) were maintained in Iscove's medium prepared with 10% FBS, 4 mM glutamine, 100 μg/ml Penicillin and 100 units/ml Streptomycin. Effector-to-target cell ratios were adjusted to 12.5:1 (2.5×10^5:2×10^4) and 25:1 (5×10^5:2×10^4) and plated in quadruplicate in 96-well, NUNC v-bottom culture plates (Fisher Scientific) containing effector cells (2.5×10^5 and 5×10^5) and target cells (2×10^4). Following incubation for 6 h at 37°C in a humidified CO_2 incubator, lactate dehydrogenase (LDH) release by lysed target cells was measured spectrophotometrically at 490 nm using a Ceres 900 microtiter plate reader (Bio-Tek Instrument, Inc., Winooski, VT). Percent cytotoxicity was calculated using the mean for each quadruplicate in the following equation: [(E – LS – TS) / (TM – TS)] x 100, where E = experimental wells, LS = lymphocyte spontaneous LDH release, TS = target spontaneous LDH release, and TM = target maximum LDH release.

Statistical Analysis

Statistical analyses were performed in collaboration with the Statistical Consulting Center at Virginia Tech. Changes in the immune parameters measured were statistically analyzed between the two treatment groups and over time using a repeated measure ANOVA (Sigma Stat, SPSS Inc., Chicago, IL). Mean differences were considered to be significant at $p<0.05$. All data are expressed as means ± SEM.

Results

Subjects

Participant information is given in the Table 1. All female subjects recruited for this study were Caucasian, age 50-69 y. Mean ages for the Suppl group (59.0 ± 1.7 y) and the Ctrl group (57.1 ± 2.0 y) were similar (Table 3.1). All participants were considered healthy and had been postmenopausal for ≥ 2 years.

According to the 3-day dietary records, all 18 subjects avoided eating any products containing soy isoflavones. Subject compliance was high, as reflected by the biochemical assessment of plasma isoflavone concentrations as well as the initialed calendars and phone interviews. The plasma concentration of the primary soy isoflavone, genistein, was significantly ($p<0.005$) higher in the supplemented group compared to the control group after 28 d of soy treatment (Table 3.1). None of the women who participated in the study reported any adverse effects from the soy isoflavone supplementation.

Table 3.1: Subject Characteristics

Age (y)	N	Age (y)	Years Menopausal	Plasma Genistein (nmol/L)[†]	Plasma Daidzein (nmol/L)[†]
Control	9	57.1 ± 2.0	6.6 ± 2.7	ND	ND
Supplemented	9	59.0 ± 1.7	7.1 ± 2.1	402.4 ± 121.0*	ND

Values are expressed as means ± SEM. ND= none detected
[†] N=8 for plasma isoflavone concentration data.
*Values are significantly different between the control group and supplemented group at $p<0.005$.

Peripheral Lymphocyte Distribution

Flow cytometric analyses showed that 4 wk dietary soy isoflavone supplementation had no significant effect on peripheral lymphocyte composition. These data are shown in Table 3.2 and Figures 3.1-3.3. Circulating lymphocyte subpopulations were similar between the two groups at baseline and did not change significantly in response to the isoflavone treatment. Mean percentages for all measured lymphocyte subpopulations fell within normal ranges for healthy adult females.

Table 3.2: Distribution of circulating lymphocyte subpopulations expressed as percentages of total gated lymphocytes at baseline (pre) and after 4 wk (post) supplementation with placebo (Ctrl) or 100 mg/d soy isoflavone tablets (Suppl).

Cell Type	Ctrl Pre (%)	Ctrl Post (%)	Suppl Pre (%)	Suppl Post (%)
$CD3^+CD4^+$	43.4 ± 2.4	44.9 ± 2.4	43.8 ± 1.8	49.0 ± 4.3
$CD3^+CD8^+$	26.2 ± 1.9	24.1 ± 1.8	25.0 ± 2.3	24.7 ± 2.1
$CD3^+$	64.5 ± 2.6	65.0 ± 2.6	68.3 ± 2.8	65.8 ± 3.1
$CD19^+$	10.8 ± 1.1	10.5 ± 1.1	9.3 ± 1.0	9.1 ± 1.0
$CD16^+CD56^+$	12.7 ± 2.1	11.4 ± 1.8	10.3 ± 1.8	10.6 ± 2.2
$CD3^+CD4^+/CD3^+CD8^+$	1.7 ± 0.2	1.9 ± 0.2	1.9 ± 0.2	2.1 ± 0.3
$CD3^+/CD19^+$	6.8 ± 2.6	7.4 ± 2.6	8.4 ± 2.8	8.6 ± 3.0

Values are means ± SEM, $p>0.05$ between groups and time.

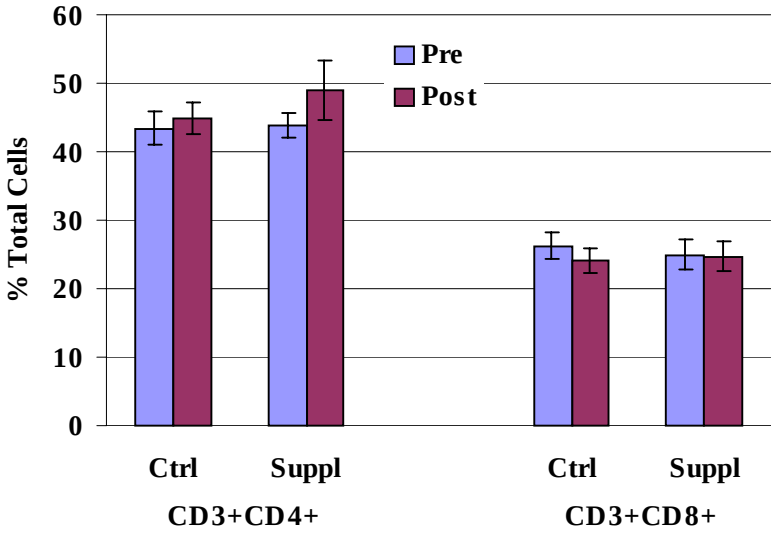


Figure 3.1: Distribution of $CD4^+$ (T-helper) and $CD8^+$ (T-cytotoxic) cells expressed as percentages of total gated circulating lymphocytes at baseline (pre) and after 4 wk (post) supplementation with placebo (Ctrl; n=7) or 100 mg/d soy isoflavone tablets (Suppl; n=9). Values are means ± SEM, $p>0.05$ between groups.

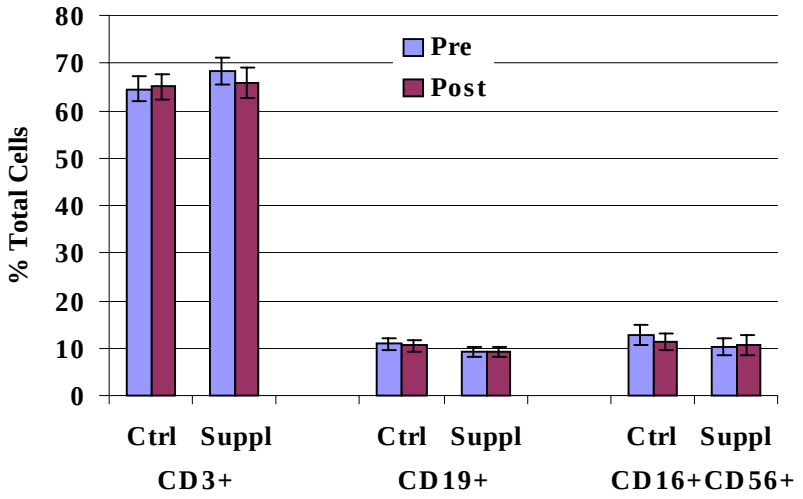


Figure 3.2: Distribution of $CD3^+$ (pan-T), $CD19^+$ (pan-B) and $CD16^+$, $CD56^+$ (natural killer) cells expressed as percentages of total gated circulating lymphocytes at baseline (pre) and after 4 wk (post) supplementation with placebo (Ctrl; n=9) or 100 mg/d soy isoflavone tablets (Suppl; n=9). Values are means ± SEM, $p>0.05$ between groups.

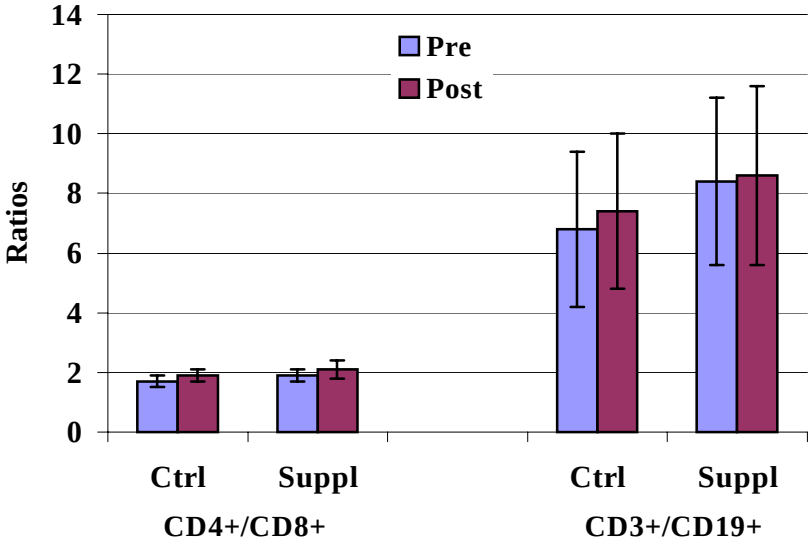


Figure 3.3: Ratio of T-helper/T-cytotoxic ($CD3^+CD4^+/CD3^+CD8^+$) cells and pan-T/pan-B ($CD3^+/CD19^+$) cells at baseline (pre) and after 4 wk (post) supplementation with placebo (Ctrl; n=7 for $CD3^+CD4^+/CD3^+CD8^+$, n=9 for $CD3^+/CD19^+$) or 100 mg/d soy isoflavone tablets (Suppl; n=9). Values are means ± SEM, $p>0.05$ between groups.

Lymphocyte Proliferation

Measurement of ^{3}H-thymidine incorporation in Con A-stimulation of lymphocyte cultures was used to study proliferation changes in response to soy isoflavone supplementation. Data are expressed as stimulation index, which is calculated as the average counts per minute (cpm) of mitogen stimulated cultures minus the cpm of cultures without mitogen (baseline cpm) divided by the baseline cpm. Proliferative ability of lymphocytes in response to the mitogen was unaltered after soy supplementation. At Con A concentrations of 2.5 µl/ml, the mean stimulation index for the Ctrl (pre=85.60; post=78.71) and Suppl (pre=70.92; post=70.35) groups did not differ significantly (Fig 3.4). An increase in the stimulation index was observed at the higher Con A concentration (5.0 µl/ml), however the indices did not differ significantly between the Ctrl (pre=134.71; post=105.76) and Suppl (pre=90.30; post=101.79) groups (Fig 3.4).

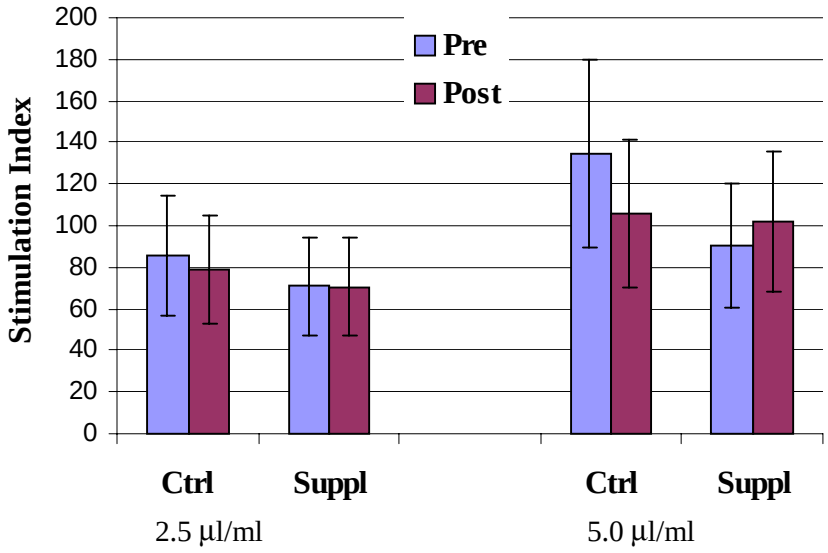
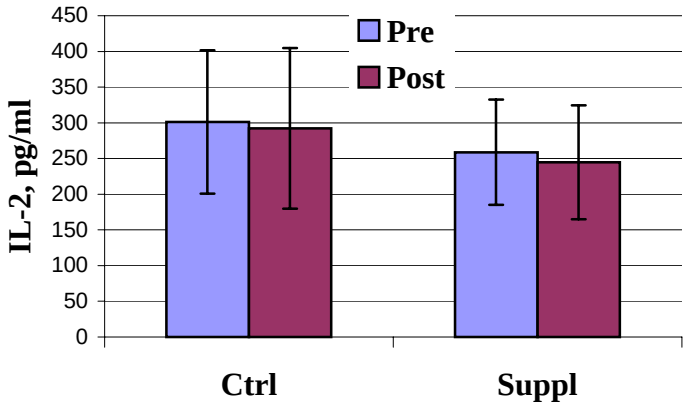


Figure 3.4: ^{3}H thymidine incorporation by Con A stimulated lymphocytes isolated from subjects (n=9/group) at baseline (pre) and 4 wk (post) supplementation with placebo (Ctrl) or 100 mg/d soy isoflavones (Suppl) for 4 wk. Values are means ± SEM, $p>0.05$ between groups.

Cytokines IL-2 and IFN Production

Concentrations of IL-2 and IFNγ produced by Con A stimulated lymphocytes did not differ significantly between the two treatment groups after 4 wk of soy supplementation. Mean baseline IL-2 concentrations for Ctrl and the Suppl groups were; 301.18 pg/ml and 258.83 pg/ml respectively, while the corresponding post-intervention levels were 292.19 pg/ml and 244.81 pg/ml respectively (Fig 3.5A). IFNγ concentrations for the Ctrl group were pre=1053.84 pg/ml; post=754.75 pg/ml and for the Suppl group IFNγ levels were pre=923.83 pg/ml; post=642.13 pg/ml (Fig 3.5B).

(A)



(B)

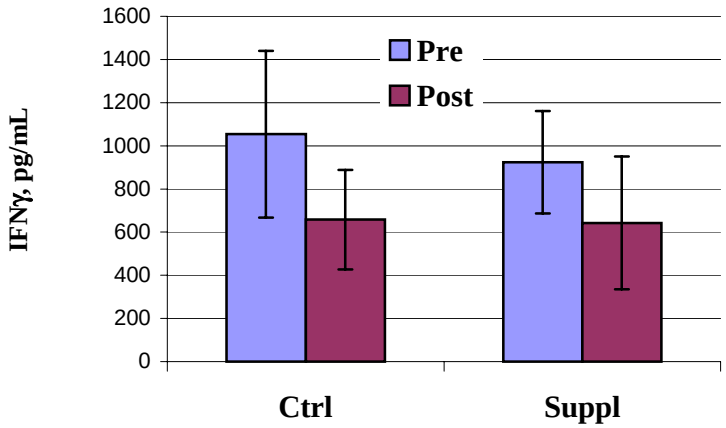


Figure 3.5: Cytokine production by Con A stimulated (10 µg/ml) lymphocytes isolated from subjects at baseline (pre) and 4 wk (post) supplementation with placebo (Ctrl) or 100 mg/d soy isoflavone tablets (Suppl) for 4 wk. **(A)** IL-2 (Ctrl n=8; Suppl n=6) and **(B)** IFNγ (n=5/group) concentrations were assessed by ELISA. Values are means ± SEM, $p>0.05$ between groups.

Natural Killer Cell Activity

No significant differences were observed in NK cell-mediated cytotoxicity in response to soy isoflavone supplementation for 4 wk. NK (effector) cell activity was based on LDH release by K562 (target) cells at 25:1 and 12.5:1 effector-to-target cell ratios. At the higher ratio (25:1), mean NK cell cytotoxicity was 24.6 ± 2.9 % and 26.1 ± 3.6 % (pre) and 22.5 ± 2.1 % and 19.5 ± 3.0 % (post) for the Ctrl and Suppl group, respectively (Fig 3.6). At the lower ratio (12.5:1), mean % cytotoxicity was 16.9 ± 3.8 and 17.1 ± 5.4 (pre) and 13.3 ± 2.9 and 12.4 ± 5.3 (post) for the Ctrl and Suppl group, respectively (Fig 3.6).

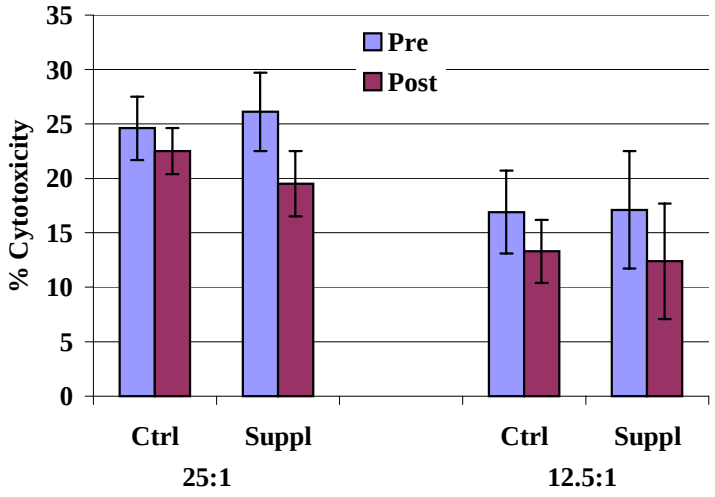


Figure 3.6: Percent cytotoxicity of circulating natural killer cells based on lactate dehydrogenase release by target K562 cells at baseline (pre) and after 4 wk (post) supplementation with placebo tablets (Ctrl; n=9 for 25:1, n=7 for 12.5:1) or 100 mg/d soy isoflavone tablets (Suppl; n=8 for 25:1, n=5 for 12.5:1). The ratios 25:1 and 12.5:1 represent the two effector-to-target cell ratios used. Values are means ± SEM, $p>0.05$ between groups.

Discussion

A growing body of evidence suggests that nutrition and immune function are closely related. In recent years the soy isoflavones, genistein and daidzein, have received increasing attention due to their potential to prevent a variety of cancers, cardiovascular disease, osteoporosis and their role in alleviating menopausal symptoms. These protective actions have been attributed, in part, to the weak estrogenic activity of isoflavones as well as estrogen-independent mechanisms such as antioxidant, antiproliferative, anti-inflammatory, enzyme inhibitory and differentiation-inducing properties. Yet, to date, research examining the effects of soy isoflavone supplementation on immune parameters in humans is limited. Since aging has been associated with decreased immunocompetence, we studied the effects of soy isoflavones on cell-mediated immunity in postmenopausal females. In this study, we found no difference in the distribution of circulating lymphocytes, the *in vitro* proliferative ability of lymphocytes in response to Con A stimulation, the production of cytokines, and the activity of natural killer cells after 4 wk of soy isoflavone supplementation at 100 mg/d in healthy postmenopausal women.

The results of several *in vitro* and animal studies have suggested enhanced immune function in response to soy isoflavone supplementation. In an *in vitro* study conducted by Wang and colleagues (10), daidzein at physiologically relevant concentrations (0.01-10 μM) in culture medium potentiated lymphocyte activation and increased IL-2 and IL-3 production in mitogen-activated murine lymphocyte cultures. However, in our study, 100 mg/d of soy isoflavone supplementation, which resulted in mean plasma genistein concentration of 0.4 μM and no detectable plasma daidzein concentration, did not significantly alter lymphocyte proliferation or cytokine IL-2 and IFNγ production in response to stimulation by the mitogen Con A. In an earlier *in vivo* study (24), the same researchers reported an enhancement of lymphocyte activation in response to high doses of daidzein supplementation (20-40 mg/kg/d) in mice. However, this immunostimulatory effect of daidzein was not observed at the physiologically relevant concentration of 10 mg/kg (24). A major difference between the previous studies and our *in vivo* human study was that the former studies used daidzein exclusively, whereas the soy isoflavone tablets used in this intervention trial mimicked the natural isoflavone composition of soybeans. In addition, unlike our investigation, the study of Wang and colleagues (10) was *in*

vitro and thus differences in bioavailability of the isoflavones could have been a decisive factor in determining our results.

In this study, 4 wk of soy isoflavone supplementation did not affect NK cell-mediated K562 cancer cell killing. These findings were in contrast to data obtained by Zhang and colleagues (11), where genistein significantly stimulated human NK cell-induced K562 cell lysis at a concentration range of 0.1-0.5 μM. Yet at higher concentrations (5-50 μM) significant inhibition of NK cell-mediated cytotoxicity was observed. Although the mean genistein concentration of 0.4 μM among our supplemented subjects falls between the two lower concentrations (0.1 and 0.5 μM) used by Zhang and coworkers (11), the lymphocytes in our lab had no direct exposure to soy isoflavones during their 6 h incubation with K562 cancer cells. Therefore, if genistein is indeed capable of potentiating NK cell activity, as proposed by Zhang and colleagues (11), the presence of this isoflavone may be required during the cytolytic process. Our results suggest that this potential effect may not be sustainable after the exposure of lymphocytes to genistein has ceased. Alternatively, isoflavones may be affecting the target cells in the culture conditions utilized in the *in vitro* study by Zhang (11).

The proposed immunomodulatory actions of genistein seem to be highly dose-dependent, since at high concentrations of this isoflavone (5-50 μM), Zhang and colleagues (11) observed significant inhibition of NK cell-mediated cytotoxicity. Thus, at pharmacological doses, genistein may actually exert immunosuppressive effects in humans. This action of genistein may partly be explained by its role as a specific PTK inhibitor, since the activation of NK cell cytotoxicity is dependent on tyrosine phosphorylation (12).

Further evidence that soy isoflavones may suppress immune function comes from two recent animal studies. O'Connor and colleagues (15) fed Lewis rats diets containing high doses of soy isoflavones (5 mg/g isoflavones) as immunosuppressive agent to delay rejection of rat cardiac allografts. In addition, genistein (5 mg/d for 21 d) intravenously injected into ovariectomized female mice resulted in an 80% reduction of thymic weight, while genistein injection at 2 mg/d for only 7 d led to a 70% decrease in thymic weight (14). The researchers

also found that the proportions of thymic $CD4^+$ and $CD4^+CD8^+$ cells decreased in genistein treated mice compared to the control animals (14).

As previously mentioned, we report no significant changes in the specific immune parameters, including NK cell activity and lymphocyte distribution. In the above studies, the inhibitory activity of genistein was observed only at concentrations substantially higher than the plasma genistein levels (0.2-1.2 μM) obtained in our study. Furthermore, the routes of isoflavone administration used in the previous studies were direct isoflavone injection or consumption of diets containing high isoflavone soy proteins (5 mg/g isoflavones) (15). On the contrary, in the present study, subjects ingested tablets containing concentrations of isoflavones (100 mg/d) typically found in Asian soy rich diets (19,20). Thus, route of administration and dose could have contributed to the observed results. Additionally, the differences in individual bioavailability of dietary soy isoflavones may have been an important factor in our study. Since we utilized an *in vivo* approach, a number of factors could have influenced the metabolism and uptake of the ingested isoflavones. In fact, on measuring plasma isoflavones we found variations in circulating genistein levels from 0.2-1.2 μM. In addition to being dose-dependent, isoflavone-mediated activities are further influenced by a number of other factors, such as endogenous estrogen levels. The fact that we did not measure plasma concentrations of 17β-estradiol may be a potential limitation of this study. However, since our subject population consisted of postmenopausal women, we did not foresee differences in estrogen levels as being a confounding factor.

In conclusion, data from our study revealed that 28 d of soy supplementation did not alter selected immunologic parameters in healthy postmenopausal women. This is important information in light of the recent data regarding the ability of the synthetic isoflavone derivative, ipriflavone, to decrease the number of circulating lymphocytes in postmenopausal women consuming this drug for 6 mo (16). Thus, further studies documenting the long-term effect of soy isoflavone supplementation on immunity are warranted.

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Chapter 4: Results

Subjects

A total of 20 postmenopausal women were recruited from the Blacksburg vicinity for participation in this study. Subjects were screened according to the following exclusion criteria: unusual dietary habits; regular consumption of soy-rich foods; high alcohol consumption; last menstrual cycle < 2 y ago; history of disease; use of hormone replacement therapy; intake of any tobacco products; and use of drugs, dietary- and/or herbal supplementation known to influence immune function. Subjects were randomly divided into two treatment groups. The supplemented group (Suppl, n=10) received two-50 mg soy isoflavone tablets/d and the control group (Ctrl, n=10) consumed two similar looking placebo tablets/d. One subject in each treatment group had consumed soy isoflavone-containing foods prior to the study, as revealed by the baseline (pre) plasma isoflavone data. Thus, a total of 18 subjects (n=9/group) were included in the statistical analyses of this trial.

Subject information is given in **Table 1**. All female subjects recruited for this study were Caucasian, ranging in age from 50 to 69 y. Mean ages for the Suppl group and the Ctrl group were 59.0 ± 1.7 y and 57.1 ± 2.0 y, respectively. All participants were considered generally healthy, as assessed by brief medical history questionnaires. All subjects had also been postmenopausal for ≥2 y. More specifically, women receiving soy isoflavone tablets (Suppl) had been postmenopausal for an average of 7.1 ± 2.1 y and those receiving the placebo tablets (Ctrl) had been postmenopausal for an average of 6.6 ± 1.1 y.

All subjects were free-living and were encouraged to maintain their usual dietary habits, which did not include the regular consumption of soy-rich foods. According to 3-d dietary records, all 18 subjects avoided eating any products containing soy isoflavones. Likewise, based on the calendars and phone interviews, compliance in taking 2 soy tablets/d was good. This self-reported information was confirmed with the biochemical assessment of soy isoflavone plasma concentrations. Baseline (pre) mean plasma concentrations of both major soy isoflavones, genistein and daidzein, were below the limits of detection for the Suppl group and Ctrl group. After the 4-wk intervention (post), the mean plasma concentration of genistein for the supplemented group (Suppl) was 402.4 ± 121.0 nmol/L, while there were no detectable amounts

of daidzein (**Table 1**). There were also no detectable amounts of either soy isoflavone found among subjects of the control group. Despite high inter-subject variability (**Table 2**), a significant increase ($p<0.005$) in mean plasma genistein concentration was observed in the supplemented group following the 4-wk intervention. None of the women who participated in the study reported any adverse effects from ingesting the tablets provided to them.

Table 1: Subject Characteristics

Age (y)	N	Age (y)	Years Menopausal	Plasma Genistein (nmol/L)[†]	Plasma Daidzein (nmol/L)[†]
Control	9	57.1 ± 2.0	6.6 ± 2.7	ND	ND
Supplemented	9	59.0 ± 1.7	7.1 ± 2.1	402.4 ± 121.0*	ND

Values are means ± SEM. ND= none detected
[†] N=8 for plasma isoflavone concentration data.
*Values are significantly different between the control group and supplemented group at $p<0.005$.

Table 2: Plasma genistein concentrations of individual subjects after supplementation with 100 mg/d of soy isoflavone tablets for 4 wk.

Subject No.	Plasma Genistein (nmol/L)
1	532.9 ± 50.7
5	190.5 ± 3.1
7	166.2 ± 8.7
9	260.5 ± 21.6
11	1198.2 ± 39.0
12	360.2 ± 25.4
14	301.8 ± 20.6
18	208.9 ± 17.0

Values are means ± SEM.

Peripheral Lymphocyte Distribution

Flow cytometric analyses showed that 4 wk dietary soy isoflavone supplementation did not alter circulating lymphocyte subpopulations. These data are shown in **Table 3** and **Figures 2-4**. Mean values for all measured lymphocyte subpopulations fell within normal ranges for healthy adult females.

Table 3: Distribution of circulating lymphocyte subpopulations expressed as percentages of total gated lymphocytes at baseline (pre) and after 4 wk (post) supplementation with placebo (Ctrl) or 100 mg/d soy isoflavone tablets (Suppl).

Cell Type	Ctrl Pre (%)	Ctrl Post (%)	Suppl Pre (%)	Suppl Post (%)
$CD3^+CD4^+$	43.4 ± 2.4	44.9 ± 2.4	43.8 ± 1.8	49.0 ± 4.3
$CD3^+CD8^+$	26.2 ± 1.9	24.1 ± 1.8	25.0 ± 2.3	24.7 ± 2.1
$CD3^+$	64.5 ± 2.6	65.0 ± 2.6	68.3 ± 2.8	65.8 ± 3.1
$CD19^+$	10.8 ± 1.1	10.5 ± 1.1	9.3 ± 1.0	9.1 ± 1.0
$CD16^+CD56^+$	12.7 ± 2.1	11.4 ± 1.8	10.3 ± 1.8	10.6 ± 2.2
$CD3^+CD4^+/CD3^+CD8^+$	1.7 ± 0.2	1.9 ± 0.2	1.9 ± 0.2	2.1 ± 0.3
$CD3^+/CD19^+$	6.8 ± 2.6	7.4 ± 2.6	8.4 ± 2.8	8.6 ± 3.0

Values are means ± SEM, $p>0.05$ between groups and over time.

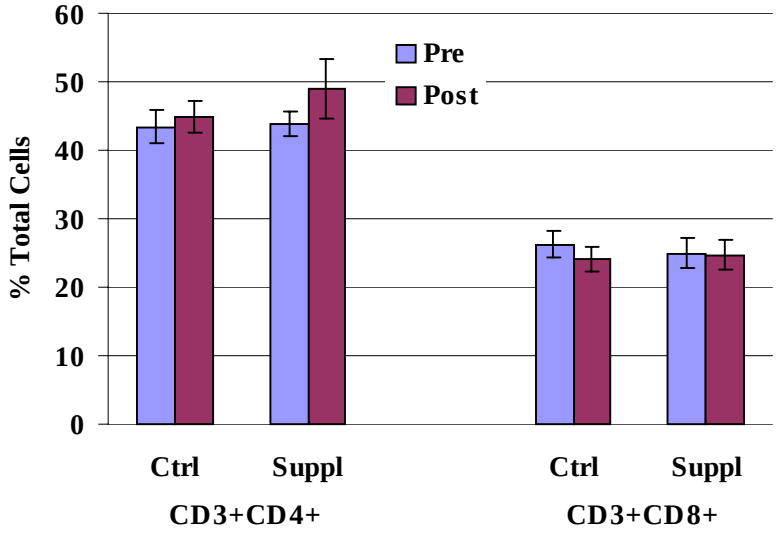

Figure 2. Distribution of $CD4^+$ (T-helper) and $CD8^+$ (T-cytotoxic) cells expressed as percentages of total gated circulating lymphocytes at baseline (pre) and after 4 wk (post) supplementation with placebo (Ctrl; n=7) or 100 mg/d soy isoflavone tablets (Suppl; n=9). Values are means ± SEM, $p>0.05$ between groups.

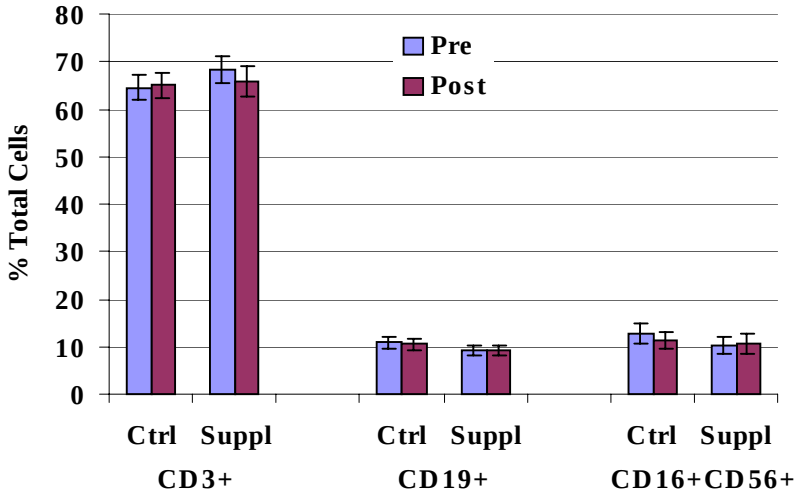

Figure 3. Distribution of $CD3^+$ (pan-T), $CD19^+$ (pan-B) and $CD16^+$, $CD56^+$ (natural killer) cells expressed as percentages of total gated circulating lymphocytes at baseline (pre) and after 4 wk (post) supplementation with placebo (Ctrl; n=9) or 100 mg/d soy isoflavone tablets (Suppl; n=9). Values are means ± SEM, $p>0.05$ between groups.

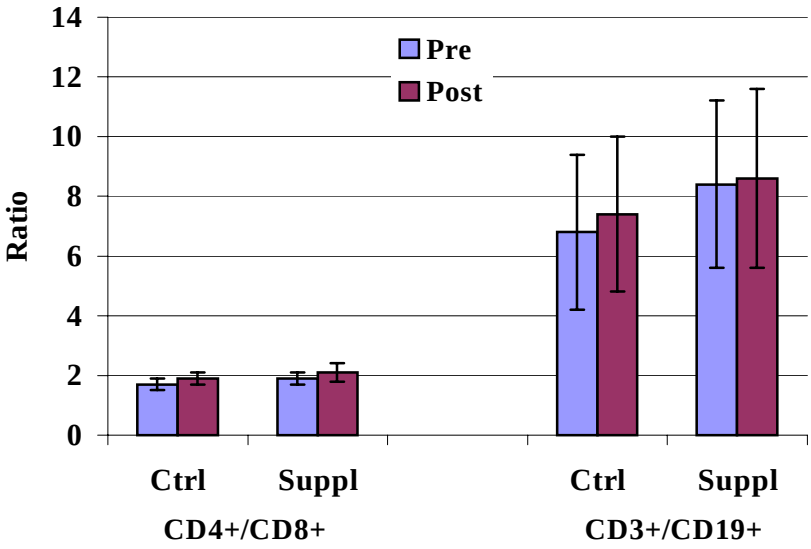

Figure 4. Ratio of T-helper/T-cytotoxic ($CD3^+CD4^+/CD3^+CD8^+$) cells and pan-T/pan-B ($CD3^+/CD19^+$) cells at baseline (pre) and after 4 wk (post) supplementation with placebo (Ctrl; n=7 for $CD3^+CD4^+/CD3^+CD8^+$, n=9 for $CD3^+/CD19^+$) or 100 mg/d soy isoflavone tablets (Suppl; n=9). Values are means ± SEM, $p>0.05$ between groups.

.

Natural Killer Cell Activity

No significant differences were observed in NK cell-mediated cytotoxicity in response to soy isoflavone supplementation for 4 wk (**Figure 5**). NK (effector) cell activity was based on LDH release by K562 (target) cells at 25:1 and 12.5:1 effector-to-target cell (E:T) ratios. At the higher ratio (25:1), mean NK cell cytotoxicity was 24.6 ± 2.9 % and 26.1 ± 3.6 % (pre) and 22.5 ± 2.1 % and 19.5 ± 3.0 % (post) for the Ctrl and Suppl group, respectively. For subjects with enough isolated lymphocytes, the assay was repeated using the lower E:T ratio of 12.5:1. Mean % cytotoxicity at this ratio was 16.9 ± 3.8 and 17.1 ± 5.4 (pre) and 13.3 ± 2.9 and 12.4 ± 5.3 (post) for the Ctrl and Suppl group, respectively.

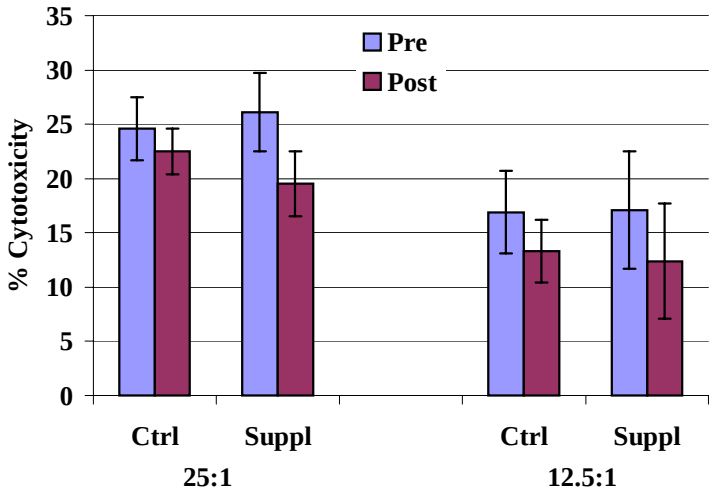

Figure 5. Percent cytotoxicity of circulating natural killer cells based on lactate dehydrogenase release by target K562 cells at baseline (pre) and after 4 wk (post) supplementation with placebo tablets (Ctrl; n=9 for 25:1, n=7 for 12.5:1) or 100 mg/d soy isoflavone tablets (Suppl; n=8 for 25:1, n=5 for 12.5:1). The ratios 25:1 and 12.5:1 represent the two effector-to-target cell ratios used. Values are means ± SEM, $p>0.05$ between groups.

Chapter 5: Discussion

A growing body of evidence suggests that sex steroid hormones, particularly 17β-estradiol, may alter immune cell function (10,18,19,20,21,22,23,24). The two primary soy isoflavones, genistein and daidzein, possess weak estrogenic activity due to their structural similarity to this female hormone (9). In addition, soy isoflavones may modulate immunocompetence due to their antioxidant, antiproliferative, anti-inflammatory, differentiation-inducing and/or lymphocyte-potentiating properties. Soy food products and soy tablets may already be widely consumed by postmenopausal women for the prevention of heart disease, osteoporosis, some types of cancers as well as alleviation of menopausal symptoms. Furthermore, as part of the natural aging process, immune function may be compromised in this population sector. Thus, we tested the hypothesis that soy isoflavone supplementation alters the distribution of circulating lymphocytes and the activity of NK cells in healthy postmenopausal women. However, according to our data, short-term soy supplementation did not change either of these indices of immunity in our target population. To date, few investigations have explored the relationship between soy isoflavone consumption and the human immune system and, to our knowledge, this was the first *in vivo* study to assess the effects of soy isoflavones on cell-mediated immunity in postmenopausal women.

NK cells play a particularly important role as anticarcinogenic agents because of their ability to kill tumor cells without first having to be activated by other lymphocytes (15). In a recent *in vitro* experiment conducted by Zhang and colleagues (55), genistein significantly stimulated NK cell-induced K562 cancer cell killing at a concentration range of 0.1-0.5 μM. Since Japanese adults consuming soy-rich diets typically have plasma isoflavone concentrations of 3-5 μM (28), it seems plausible that genistein-stimulated potentiation of human peripheral NK cells is one of the primary mechanisms responsible for enhanced immunosurveillance. However, results from our *in vivo* study suggest that soy isoflavone supplementation (100 mg/d) for 4 wk did not alter cytotoxicity of circulating human NK cells. While we performed the nonradioactive enzyme-release assay for determination of NK cell percent cytotoxicity, Zhang et al. (55) used the ^{51}Cr-release assay. Yet, this difference in methodology should not preclude a legitimate comparison of our results to those of Zhang and associates (55). According to Korzeniewski and

Callewaert (59), the enzymatic LDH assay is as accurate and precise in measuring NK cell-mediated cytotoxicity as the standard ^{51}Cr-release method, while it has the distinct advantages of being safer as well as less expensive and time-consuming.

Another fundamental difference between our study and that conducted by Zhang and colleagues (55) was the study design. In the latter study, isolated peripheral blood lymphocytes and K562 cancer cells were incubated in the presence of 0.1, 0.5, 5, 10 and 50 μmol/L genistein (55). In our laboratory, peripheral blood lymphocytes were isolated from subjects following daily consumption of soy isoflavones (100 mg) for 4 wk. While the mean genistein plasma concentration of 0.4 μmol/L (range = 0.2 – 1.2 μmol/L) among our supplemented subjects falls between the two lower concentrations used by Zhang et al. (55), the lymphocytes and K562 cells in our lab had *no direct* exposure to soy isoflavones *during* their 6 h incubation with K562 cells. Therefore, if genistein is indeed capable of potentiating NK cell activity, as proposed by Zhang and colleagues (55), the presence of this isoflavone may be required during the cytolytic process. Our results suggest that the proposed isoflavone-mediated effect may not be sustainable after the exposure of lymphocytes to genistein has ceased. Similarly, if soy isoflavones, like estrogen and tamoxifen (56), are capable of increasing the susceptibility of K562 cells to NK cell lysis, this change in target cell sensitivity also seems to require direct exposure to the modulating agent. While Baral et al. (56) pretreated both target cells and effector cells with tamoxifen and/or estradiol at various concentrations, the target cells in our lab had no direct exposure to soy isoflavones either before or during their incubation with human NK cells.

As mentioned earlier, we found no significant alterations in peripheral lymphocyte subpopulations in response to 4 wk of soy isoflavone supplementation. However, in a recent study conducted by Yellayi et al. (57), genistein injection into ovariectomized female mice resulted in decreased proportions of thymic $CD4^+$ and $CD4^+CD8^+$ cells in the treated mice compared to the control animals. This discrepancy between our results and those obtained by Yellayi et al. (57) may be explained by the fundamental differences between these two investigations. While we assessed the distribution of circulating lymphocytes in postmenopausal women after dietary isoflavone supplementation, Yellayi and colleagues (57) measured the distribution of lymphocytes directly isolated from the thymus of ovariectomized mice after

genistein was intravenously injected. Thus, our study design more accurately mimics the isoflavone exposure that would occur in humans consuming either soy isoflavone tablets or soy foods.

Compared to American women, Asian women, who typically consume soy-rich diets, have significantly lower rates of various chronic diseases. This apparent protection could be linked to enhanced immunocompetence. However, results from the present study do not support the theory that short-term soy isoflavone supplementation in free-living postmenopausal women alters lymphocyte distribution or NK cell cytotoxicity. During the 4-wk intervention, the treatment group ingested 100 mg/d of soy isoflavones in the form of 2 tablets/d. This amount of soy isoflavones is physiologically attainable and comparable to the average per capita consumption of 50-100 mg/d among Asian women in general (33,34) and the estimated 200 mg/d among Japanese women in particular (32). Data from dietary records kept by our subjects and, more importantly, measured plasma soy isoflavone concentrations indicate that overall subject compliance was high with respect to avoidance of soy isoflavone-containing foods and intake of soy supplementation. However, the range of plasma genistein concentrations among subjects in the supplemented group (Table 2) also reveals that isoflavone bioavailability varied among these women. More specifically, the highest plasma genistein concentration was more than 7-fold higher than the lowest plasma genistein concentration (Table 2). These data are consistent with results from previous studies, which have shown that isoflavone bioavailability depends in part on the rate and extent of microbial hydrolysis (35,36). Differences in isoflavone bioavailability among subjects may certainly lead to differences in the influence of these compounds *in vivo*. Therefore, it seems prudent to investigate the effect of soy isoflavones on parameters of immune function on the basis of isoflavone bioavailability.

Phytoestrogens, like genistein and daidzein, may also alter immunocompetence *indirectly* by influencing estrogen activity and/or secretion in estrogen-sensitive tissues. Zava and Duwe (54) discovered that genistein effectively inhibited the growth of ER+ and ER- breast cancer cell lines at very high concentrations ($\geq$ 10 μM), though it seemed to have the opposite effect at physiologically relevant concentrations of 1 nM-10 μM. Other researchers found an inverse relationship between soy consumption and serum estradiol concentrations in premenopausal

Japanese women (25) and in pre- and postmenopausal American and Asian women (29). This soy-induced reduction of circulating estradiol could be one of the mechanisms by which soy isoflavones exert their proposed immunomodulatory effects. However, these mechanisms would likely be sensitive to endogenous levels of circulating estrogens as well as the amount and duration of soy consumption. Therefore, using different subject populations (i.e. pre- and peri-menopausal women) and varying the dose and length of soy supplementation may yield different results than those seen in the present study.

This study, as others, has several limitations. First, after relentless recruitment efforts, we were forced to widen the age range of our subjects in order to find enough eligible participants. With a nearly 20-y difference between the youngest and oldest subjects, the menopausal status with respect to years since last menstrual cycle was also quite different. However, since all women had been postmenopausal for at least 2 y, we expected their plasma estrogen levels to be negligible. Therefore, we did not find it necessary to measure the plasma concentrations of 17β-estradiol in our target population. In addition, with an age range of nearly 20 y, the onset and duration of changes in immune defense related to the aging process may have been different among our subjects. Thus, it would have been more desirable to have a more narrow subject age range in this study.

Additional limitations of this study are associated with the use of free-living subjects. Plasma isoflavone data confirm that subject compliance was high in terms of taking the prescribed amount of soy tablets as well as avoiding soy isoflavone-containing foods. Considering that Americans typically consume very little, if any, foods rich in soy isoflavones, we believe that subject compliance would likely have been much poorer if we had asked our participants to consume soy-rich foods for 28 d instead of taking soy isoflavone tablets. However, it is not known whether the isoflavones, genistein and daidzein, are the *only* physiologically active compounds in soybeans. In addition, most of the data previously reporting a relationship between soy and immune function were specific for the isoflavone component. Thus, our choice of isoflavone supplements as opposed to soy foods was important for comparison of our data to that reported in the literature. Finally, with the use of free-living subjects we cannot be absolutely certain that all subjects refrained from consuming

micronutrients and dietary supplements believed to influence immune cell integrity and function. Although dietary records indicate that most subjects followed our instructions to avoid dietary supplements or drugs thought to affect immunity, this study did not include biochemical analyses of every micronutrient known to influence immune function.

In conclusion, data from *in vitro* and animal studies have suggested that the soy isoflavones, genistein and daidzein, may influence various indices of cell-mediated immunity. However, our results indicate that 4 wk of soy isoflavone supplementation (100 mg/d) did not alter the distribution of circulating lymphocytes or the activity of NK cells in healthy postmenopausal women. According to the 2001 Soy Food Guide, soy food sales in the United States have increased dramatically from $2 million to just over $2 billion during the past two decades (12). Thus, due to the widespread public interest in this supplement and the conflicting data concerning the effects of dietary soy isoflavones on immune function, further research in this area is warranted.

Chapter 6: Future Research

Research investigating the effects of soy isoflavone supplementation on immune function in humans is still limited. Both *in vitro* and *in vivo* data suggest that cellular mechanisms of soy isoflavones are highly sensitive to circulating estrogens, amounts of dietary isoflavones and, perhaps most importantly, the type of target tissue. Therefore, it is logical to explore how extending the soy supplementation period and varying the supplementation dosage may affect the distribution and function of peripheral lymphocytes in humans. Furthermore, targeting female subjects of different age groups and menopausal status may help to elucidate the highly complex relationship between endogenous hormone levels and soy phytoestrogens with respect to their influence on immunocompetence. These and other variables could be examined by conducting a series of clinical trails with a crossover design, which would incorporate a washout period between the intervention periods. In addition, to account for the high variability in soy isoflavone bioavailability, future human intervention trials should include enough subjects to allow for the stratification of data on the basis of plasma isoflavone concentrations.

Epidemiological data indicate that Asians who typically consume soy-rich diets have significantly lower rates of several chronic diseases. Hence, it is possible that the protective effect of soy isoflavones is manifested only when soy consumption occurs early in life (i.e. before the immune system has matured). To test this hypothesis, longitudinal studies are needed to measure the timing and nature of changes in cell-mediated immunity in response to soy consumption.

Soy supplementation is becoming increasingly popular among Americans for its potential health benefits such as reduced risk of cardiovascular disease, osteoporosis and certain types of cancer. The recently FDA approved health claim concerning soy consumption and cardiovascular health will likely lead to an even greater use of soy supplementation in this country. Given the inconsistent data in the literature regarding the consequences of isoflavones on immune parameters, it is imperative to further investigate the effects of soy isoflavone consumption on immune defense in humans.

Appendix A: Experimental Design

The study followed the design of a human double-blind, placebo-controlled intervention trial for 4 wk. Healthy postmenopausal women (n=20), who were not on hormone replacement therapy, were randomly divided into 2 treatment groups to examine whether isoflavone intake modulates parameters of human immune function. The supplemented group (Suppl; n=10) consumed two-50 mg soy isoflavone tablets/d (NovaSoy, Archer Daniels Midland, Decatur, IL) for 28 d, while the control (Ctrl; n=10) group received two placebo tablets/d.

Subjects

A total of 20 postmenopausal women (aged 50-69 y) were recruited from the Blacksburg community to serve as subjects. Resources for recruitment efforts included messages posted on the Blacksburg Electronic Village (BEV) Homepage News and vtwomen@listserve.vt.edu; announcements made at the Blacksburg Seniors Program's steering committee and breakfast meetings; visitations to local senior aerobic classes; widespread distribution of flyers; and a discussion of this study aired by a local radio station. To be eligible for participation, subjects were screened carefully according to the aforementioned exclusion criteria. Each subject completed a health history questionnaire and signed an informed consent form prior to enrollment in the study. Approval for the use of human subjects in this research was granted from the Virginia Tech Institutional Review Board (IRB 99-209).

Blood Samples

Overnight fasting blood samples of 24 ml were drawn between 8:00 – 9:00 am by an experienced phlebotemist at baseline (d 1) and on d 28 of the study. Whole blood was used for flow cytometric analysis of circulating lymphocyte populations. For determination of NK cell cytotoxicity (CytoTox 96 Assay, Promega Corporation, Madison, WI), peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood using Vacutainer BD test tubes containing Ficoll-Paque reagant (BD PharMingen, San Diego, CA). Plasma was stored at –80^{o}C and assayed for isoflavone concentration, via reverse-phase high performance liquid chromatography (HPLC), after determinations of lymphocyte distribution and NK cell activity were completed.

Intervention

For 4 consecutive wk, 10 of the 20 participants were randomly selected to consume soy tablets (Archer Daniels Midland) containing a daily total of 100 mg of isoflavones in the glycone form. The remaining 10 subjects (Ctrl) were given similar looking placebo tablets for daily consumption throughout the same 4-wk period. Soy isoflavone and placebo tablets were disseminated among subjects in 4 small envelopes, each containing a 1-wk supply, on d 1 of the study following the baseline blood draw. All participants were instructed to take their dose of 2 tablets/d at the same time each morning. In addition, all subjects were urged to continue their usual dietary habits and physical activities throughout this trial.

To stress the importance of compliance, all participants were given a calendar to be initialed each day following the ingestion of both tablets. Furthermore, subjects were contacted on a regular basis during the study to minimize the potential for missed tablets or blood draws. Any participant who lost one or more tablet(s) was supplied with the appropriate number and type of replacement tablet(s). In order to monitor nutrient intake, all subjects were asked to complete two 3-d dietary intake records on specified days of weeks 2 and 4 during the intervention. The calendar, 3-d dietary records, and any remaining tablets were collected from each subject at the end of the study to further assess compliance.

Appendix B: Materials and Methods

Determination of Isoflavone Concentration in Plasma

Reverse-phase high performance liquid chromatography (HPLC) was used to quantify the plasma concentrations of genistein and daidzein at baseline (d 1) and at the end of the trial (d 28). In performing this procedure, 500 µl of plasma was diluted in 10 ml of 100% HPLC grade methanol, followed by the addition of 10 µl of the internal standard Fluorescein (500 ng = 10 µl of stock 1 mg/10 ml of 80% HPLC grade methanol). All samples were vortexed and centrifuged at 3000 x g for 10 min at 10^{o}C. After the methanol supernatant was poured off into a rotary evaporator flask, the plasma residue was resuspended two additional times in 5 ml of 100% HPLC grade methanol and then vortexed and centrifuged as above. Using a rotary evaporator, the collected methanol supernatant was evaporated, leaving the residue to be redissolved in 5 ml of 1 M sodium acetate with an additional 2 ml of 1 M sodium acetate to further rinse the flask. The enzyme glucuronidase/sulfatase (50 µl, H2 Type, Sigma G0876) was then added prior to incubating all samples in a dry heating block overnight for 19 h at 37^{o}C.

Using this hydrolysate, the isoflavones genistein and daidzein were extracted on a C18-ODS SPE reverse phase column (Fisher Scientific, Pittsburgh, PA). The columns were activated with 5 ml of 100% HPLC grade methanol and then rinsed with 5 ml of HPLC grade water. Next, the hydrolyzates were added and also pulled through the columns over ca. 2 min. For complete removal of the hydrolyzate, 1 ml of 1 M sodium acetate buffer (pH 5.5) was used to rinse each empty sample test tube. The buffer was then pulled through the corresponding column. All samples were rinsed with 2 ml of HPLC grade water before the soy isoflavones were eluted (over ca. 2 min) with 2 ml of 100% HPLC grade methanol and collected in 15 ml conical centrifuge tubes. Using nitrogen gas, the collected isoflavone/methanol fraction was brought to complete dryness, redissolved in 150 µl of 80% HPLC grade methanol and vortexed. Each sample was transferred into an HPLC vial and 100 µl was injected onto a C18 250 x 4.6 mm column (Luna, Phenomenex, Torrance, CA) for analysis. The mobile phase consisted of 15% HPLC grade methanol in HPLC grade water. All samples were run in duplicate. A standard curve was prepared using a series of genistein standards (200, 500, 1000 and 1200 ng) dissolved in 80% HPLC methanol and assayed to quantify the isoflavones.

Determination of Circulating Lymphocyte Distribution

Lymphocyte subpopulations were assessed using fluorescently labeled monoclonal antibodies (mAb) against cell surface markers. The mAb (Becton Dickinson, San Jose, CA) included: T-helper cells ($CD3^+CD4^+$, clone SK3), T-cytotoxic cells ($CD3^+CD8^+$, clone SK1), total T lymphocytes ($CD3^+$, clone SK7), total B lymphocytes ($CD19^+$, clone 4G7), and NK cells ($CD16^+CD56^+$, clones B73.1 and MY31, respectively).

The following procedure for labeling lymphocytes in lysed whole blood was applied: 20 µl of the appropriate mAbs was aliquoted into labeled test tubes, followed by 100 µl of well mixed anticoagulated (EDTA) whole blood from each subject. All samples were vortexed at low speed for 3 sec and then incubated at room temperature in the dark for 30 min. For the lysing and washing procedure, 2.0 ml of 1 x lysing solution (FACS Lysing Solution) at room temperature was added to each test tube containing the whole blood/mAbs mixture. All samples were vortexed again at low speed for 3 sec, incubated at room temperature in the dark for 10-12 min, and centrifuged at 300 x g for 5 min at 20-25^{o}C. Supernatants were aspirated, leaving approximately 50 µl of residual fluid, and vortexed again at low speed to resuspend the cell pellet. All tubes were kept on ice and the cells were washed with phosphate-buffered saline (PBS) plus 0.1% sodium azide (NaN_3) and centrifuged at 200 x g for 5 min at 20-25^{o}C. Following removal of the supernatant, pellets were resuspended in 0.5 ml of 1.0% paraformaldehyde and kept at 4^{o}C until analysis.

Samples for each subject were assayed in duplicate. Aliquots of CD-Check Plus (Becton Dickinson) were stained with mAb to serve as control for labeling. All stained blood samples were analyzed the next morning using an Epics XL Flow Cytometer (Coulter Electronics, Hialeah, FL) at the Virginia-Maryland Regional College of Veterinary Medicine Flow Cytometry Lab.

Determination of Natural Killer Cell Cytotoxicity

Lymphocyte Isolation

For isolation of circulating lymphocytes, heparinized blood from each subject was drawn into Vacutainer BD test tubes containing Ficoll Paque reagant (BD PharMingen). Isolated lymphocytes were resuspended in complete medium (RPMI supplemented with 10% autologous serum, 2 mM glutamine, 100 µg/ml Penicillin and 100 units/ml Streptomycin).

Lymphocyte (Effector Cell) Counting

Isolated lymphocytes (50 µl) were mixed with 50 µl of Tryphan Blue Dye (TPB, GIBCO) and 100 µl RPMI. Cells were then counted by loading 10 µl of the mixed cell/TPB suspension onto each chamber of a hemocytometer. Remaining lymphocytes for each subject were transferred into 15 ml conical tubes, centrifuged at 400 x g for 10 min and resuspended at a concentration of $5x10^6$ cells/ml in assay medium [Iscove's prepared with 5% fetal bovine serum (FBS), 4 mM glutamine, 100 µg/ml Penicillin and 100 units/ml Streptomycin]. Addition of 0.5 ml of assay medium to 0.5 ml of prepared $5x10^6$ cells/ml rendered the second concentration of lymphocytes ($2.5x10^6$ cells/ml) used in the NK cell cytotoxicity assay.

K562 (Target Cell) Maintenance

The K562 human myelogenous leukemia cell line (American Type Culture Collection, Manassas, VA) was used as target cells for the NK cell cytotoxicity assay. The K562 cells were maintained at 37^oC in a humidified CO_2 incubator (NuAire, Plymouth, MN) in Iscove's medium supplemented with 10% FBS, 4 mM glutamine, 100 µg/ml Penicillin and 100 units/ml Streptomycin in a T75 flask containing 25 ml media. Once the cells reached a density of 1×10^6 cells/ml, a new culture was started by re-seeding aliquots with new media at a concentration of 1×10^5 cells/ml.

K562 (Target Cell) Counting

Target cells were counted (200 µl of mixed cell suspension and 200 µl of Tryphan Blue Dye) and resuspended at a concentration of $2x10^5$/ml to be used for the NK cell cytotoxicity assay.

Enzyme Release Assay for NK Cell Cytotoxicity

NK cell cytotoxicity was measured according to the nonradioactive procedure by Korzeniewski and Callewaert (59) using the CytoTox 96 Kit (Promega Corporation). K562 cells (100 µl) at a concentration of $2x10^5$/ml and isolated lymphocytes (100 µl) at concentrations of $5x10^6$/ml and $2.5x10^6$/ml were aliquoted into NUNC v-bottom culture plates (Fisher Scientific) to yield 25:1 and 12.5:1 effector-to-target cell ratios, respectively. The plate was centrifuged at 270 x g for 4 min and then incubated for 6 h at 37°C in a humidified CO_2 incubator (NuAire). During the last 45 min of incubation, 20 µl of lysis buffer was added to wells measuring maximum lactate dehydrogenase (LDH) release by target cells, while 10 µl of lysis buffer was added to wells measuring volume control of lysis buffer addition. Following incubation, the plate was centrifuged again at 270 x g for 4 min and 50 µl aliquots were transferred into a 96-well flat-bottom plate. Assay buffer/substrate solution was heated to ca. 37°C and 50 µl aliquots were added to each well. The plate was incubated for 30 min at room temperature, protected from light. Stop solution (50 µl) was added to each well and absorbancies were read at 490_{nm} using a Ceres 900 microtiter plate reader (Bio-Tek Instrument, Inc., Winooski, VT).

This nonradioactive cytotoxicity assay (CytoTox 96, Promega Corporation) spectrophotometrically measured LDH, an enzyme that is released spontaneously upon cell death. The color intensity is directly proportional to the number of lysed cells. Therefore, this procedure determined NK cell cytotoxicity based on LDH released by lysed target cells. All samples were assayed in quadruplicate and corrections were made by running controls for the spontaneous LDH release from K562 cells and lymphocytes, contributions to absorbance at 490_{nm} by culture medium background and volume increase caused by adding lysis solution. In addition, bovine heart LDH was used as a positive control to verify that the assay was working. Percent cytotoxicity was calculated using the mean of each quadruplicate in the following

equation: [(E – LS – TS) / (TM – TS)] x 100, where E = experimental wells, LS = lymphocyte spontaneous LDH release, TS = target spontaneous LDH release, and TM = target maximum LDH release.

Statistical Analysis

Statistical analyses were performed in collaboration with the Statistical Consulting Center at Virginia Tech. Changes in soy isoflavone plasma concentrations, peripheral lymphocyte distributions, and percent cytotoxicity of NK cells were statistically analyzed between the two treatment groups and over time using a repeated measure ANOVA (Sigma Stat, SPSS Inc., Chicago, IL). Mean differences were considered to be significant at $p<0.05$. All data are expressed as means ± SEM.

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Vita

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Nicolin Girmes-Grieco, daughter of Hannes and Gisela Girmes, was born June 21, 1968, in Krefeld, Germany. At the age of 12 years, she immigrated to the United States with her family. She was conferred a Bachelor of Arts degree in International Studies by the University of North Carolina at Chapel Hill in May 1990. For the next five years, Nicolin worked in Washington, D.C., as the program director of the nonprofit environmmental organization Renew America. In her pursuit of a new career, she finished her dietetics course requirements and began the graduate program in Nutrition at Virginia Tech. During her graduate study, she received financial support as a teaching assistant from the Department of Human Nutrition, Foods and Exercise while maintaining a 4.0 GPA. She became a member of two honor societies, Kappa Omicron Nu (KON) and Kappa Phi Kappa, and served as vice president and president of KON. In July 1998 Nicolin married Stephen Grieco and in October 2000 their son Maximilian Thomas was born.