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NUTRITION AND IMMUNE FUNCTION IN HEALTHY OLDER WOMEN

A Thesis in Nutrition

by

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ABSTRACT

Aging is often associated with a dysregulation in the immune system. A decline in immunocompetence with advancing age, particularly in T cell functions, has been reported, even in healthy older adults. Nutrition is important for maintaining optimal immune function, as both macronutrients (energy and protein) and micronutrients (Vitamins A, E, B_6 and C, iron, and zinc) can influence immune function outcomes. Nutrient deficiencies can, therefore, contribute further to the age-related decline in immune function in older individuals, and can increase the risk of infections and disease.

Few studies have comprehensively examined the impact of nutrition on immune function in the healthy older adults. Most of these studies focus on correlations between levels of certain nutrients and immune response or the effect of nutrient supplements on immune function. Thus, literature on whether certain nutrients, examined concurrently, can predict immune function in older adults is very limited. Because several nutrients can influence immune function, and there are interactions between several nutrients, determining the interactive effects of nutrients on immune function is important. Therefore, we examined the relationship between specific nutrients, known to be involved in maintaining immune response, protein, iron, zinc, vitamin B_{12}, and folate, with tests of acquired immune function in healthy older women (Chapter 2). Older women (> 60y; n=130) were recruited with assistance of the Agencies on Aging from three counties and from local housing complexes for seniors. We used discriminant analysis to identify the predictive subset of nutrients, which can correctly classify women as low and high responders on tests of acquired immune response namely, T cells and subsets, lymphocyte proliferation response to phytohemagglutinin A (PHA) and concanavalin A (Con A), and production of interleukin (IL)-1β, IL-2 and IL-6. Protein and iron status variables were identified in the predictive subset for all immune function variables examined; zinc emerged in the final predictive subset for T cells and subsets, and lymphocyte proliferation response to Con A. Vitamin B_{12} and

folate were identified in the final predictive subset for only cytokine variables. The probability of correctly classifying women into low or high responders of immune function tests by the predictive subset of nutrition variables was high and ranged from 62.8-83.5% for T cells and subsets, 79.3-89.7% for lymphocyte proliferation response, and 77.8-88.9% for cytokines. Thus, this study shows that several nutrients namely iron, protein and zinc are significant predictors of immune function in an older cohort and maintaining the status of these nutrients may help maintain immunity in older adults.

The associations between the triad of nutrition, immune function and aging are influenced by a variety of factors in study design such as health status of participants, gender, and several methodological issues concerning assessment. To establish relationships between immune function and variables of interest, it is important to determine these variables accurately and precisely. Precision relates to the amount of variability in the laboratory test. The nature and magnitude of variation in immune function tests has not been described extensively. Therefore, we examined inter- and intra-individual variation in tests of cell-mediated immunity (CMI) in generally healthy and well-nourished young (20-40y; n=15) and old (60-80y; n=15) women (Chapter 3). Subjects provided blood samples on two days within a week to determine leukocyte subsets, T-cell proliferation response to PHA and Con A, and IL-1β, IL-2 and IL-6 production. Intra-individual variation was partitioned into day-to-day biological and analytical variation. Inter-individual variation was greater than intra-individual variability for all tests of CMI for both age groups. Furthermore, in both groups, all CMI tests exhibited large day-to-day intra-individual variation (CV~15% or greater), which was primarily due to biological rather than analytical sources. In conclusion, both age groups showed large between-person and considerable within-person variation in CMI tests. Therefore, repeated blood sampling to determine immune function tests can improve precision of these measurements.

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CHAPTER 1

INTRODUCTION

1.1 The immune system

The immune system is a remarkable network of organs, cells, and molecules, which help to defend the body from invading pathogens and cancer. Functionally, the cells and molecules of the immune system are capable of mounting an immune response through two inter-related activities – recognition and response. The two broad components of the immune system include non-specific or innate immunity and specific or acquired immunity (Kuby, 1997; Myrvik, 1999).

Innate immunity is comprised of four types of defensive barriers: anatomic, physiologic, endocytic and phagocytic, and inflammatory. Anatomic and physical barriers, the skin and the mucous membranes, are the body's first line of defense and provide an effective barrier to the entry of many pathogens. Physiologic barriers include temperature, pH, oxygen tension, and various soluble factors. Temperature inhibits the growth of certain pathogens in many species, and gastric acidity is effective against microorganisms as very few survive low pH of the stomach. Soluble factors such as lysozyme, which is a hydrolytic enzyme capable of cleaving bacterial cell wall, or complement, which is group of serum proteins that participate in an enzyme cascade to destroy membranes of pathogens or facilitate their clearance, also contribute to innate immunity. Endocytosis, phagocytosis, microbial killing activity of phagocytic cells such as neutrophils, monocytes, and macrophages, and destruction of tumor cells by natural killer (NK) cells are also part of innate immunity, although these non-specific effector cells are activated by cell-mediated immune responses. Inflammation is another defensive barrier that includes vasodilation, increased capillary permeability and influx of phagocytes during invasion by microorganisms, and it is initiated by complex interactions involving chemical proteins such as acute-phase proteins, kinins and histamine (Kuby, 1997).

Acquired immunity develops when the body is capable of recognizing and selectively eliminating microorganisms and molecules once it is exposed to the microorganisms or foreign

molecules. It does not occur independently of the innate immunity. Acquired immunity shows four distinct features: specificity, diversity, memory and self/nonself recognition. The two components of acquired immunity are humoral and cell-mediated immunity, and they involve two major populations of lymphocytes. Humoral immunity consists of interactions of B lymphocytes (B cells) with antigens, and subsequent proliferation and differentiation into antibody (Ab)-secreting plasma cells, whereas cell-mediated immunity involves generation of effector T lymphocytes (T cells), namely T helper cells and cytotoxic T lymphocytes, in response to antigens (Kuby, 1997). The development of an effective immune response is generated by complex interactions involving the innate and acquired immunity. Cells involved in these interactions are lymphoid cells, inflammatory cells and hematopoeitic cells. In addition, these complex immune interactions are mediated by low-molecular weight proteins called cytokines, which are secreted by a variety of immune cells, and mainly by T helper cells. Cytokines regulate the intensity and duration of an immune response by stimulating or inhibiting the activation, proliferation, and/or differentiation of the cells that produce them or other cells; and they regulate the secretion of antibodies or other cytokines by those cells (Kuby, 1997).

Various laboratory tests have been developed over the past few decades to measure innate and acquired immune function components. Aspects of innate immunity can be measured *in vitro* by quantifying phagocytosis and oxidative burst capacity of neutrophils or macrophages. Phagocytosis involves the engulfing of microorganisms by the phagocytic cells, and many antimicrobial and cytotoxic substances produced by these macrophages bring about destruction of the engulfed microorganism by oxygen-dependant and oxygen-independent killing mechanisms (Kuby, 1997; Myrvik, 1999). Phagocytosis can be quantified by flow cytometry or direct visualization of the ingested particles using light or electron microscopy, microbiological assays, the nitroblue tetrazolium test, or quantitation of Ab- and antigen (Ag)-mediated phagocytosis (Hampton and Winterbourn, 1999).

Aspects of acquired immunity can be quantified by *in vitro* tests such as plaque-forming assays to determine the number of B cells producing immunoglobulins, radial immunodiffusion for the measurement of serum immunoglobulins, flow cytometry or fluorescence microscopy for the enumeration of lymphocyte populations, proliferation response of lymphocytes to mitogens, chromium release assay for measuring NK cell and T cytotoxic cell activity etc. *In vivo*, cell-mediated immunity can be measured by the delayed-type hypersensitivity (DTH) response, which employs use of skin test antigens such as candidin and tuberculin. The skin test response is apparent after 24-30 hours when there is an infiltration of macrophages and lymphocytes at the site (Kuby, 1997; Myrvik, 1999).

1.2 Aging and immune function

Aging has been associated with an increased susceptibility to infections and cancer, in addition to the morbidity and mortality associated with these conditions (Makinodan, 1995). Although a number of factors contribute to these conditions, the age-related changes in various components of the immune system have been thought to play a very important contributory role. This is shown in a study by Wayne et al. (1990), in which a healthy cohort of the New Mexico Aging Process (NMAP) study were followed up annually, after tests of DTH response and proliferation response to phytohaemagglutinin A (PHA), for about 10 years. Anergy (failure to respond to four skin-test antigens by DTH) was associated with an increased risk of cancer and all-cause mortality, and was a better predictor of mortality than PHA. Murasko and colleagues (1987) also studied proliferation responses of peripheral blood mononuclear cells (PBMC) from young and old subjects to three mitogens – PHA, concanavalin A (Con A), and pokeweed mitogen (PWM). They reported two important findings: a gradual decrease in mitogens proliferation response with age, and a significant association between lack of response to the three mitogens and an increase in mortality. Similar findings are seen in other studies where low NK cell activity (Ginaldi et al., 1999; Levy et al., 1991), and decreases in chemotaxis of

phagocytic cells and increase in serum lipid peroxidase levels (Niwa et al., 1989) have been shown to predict impending morbidity and mortality.

Immunogerontological studies have focused on age-related changes in immune system for several decades. Evidence from many clinical and experimental data in humans and animals suggest a decline in immune function with aging, which has been termed ‘immune senescence’. However, an important limitation in many of these studies is the absence of strict subject selection criteria pertaining to health status, such as the SENIEUR protocol (Ligthart et al., 1984). Thus, some of the age-associated immune deficiencies or declines that have been reported can be the result of disease, inflammation or infection, and not necessarily because of the aging process itself. The SENIEUR protocol is a standardized set of criteria for subject selection in immunogerontological studies based on clinical and laboratory data, which allows for selection of subjects without any underlying disease or inflammatory conditions. It was developed by the EURAGE Concerted Action Programme on Ageing of the European Community, and provides researchers with guidelines for eliminating any confounding conditions that may interfere with immune responses (Ligthart et al., 1984). Therefore, it enables selection of ’healthy’ participants.

Many of the recent studies on immune function and aging have acknowledged the use of strict selection criteria in their study design (Corberand et al., 1986; Facchini et al., 1987; Krause et al., 1999; Lesourd et al., 1998; Sansoni et al., 1993). Using the SENIEUR protocol, studies have reported fewer age-related differences in immunocompetence (Corberand et al., 1986; Facchini et al., 1987). However, some age-related declines or differences are still noted with the SENIEUR protocol (Oyeyinka et al., 1995; Polignano et al., 1994). Therefore, it may be possible that other factors such as nutritional status, particularly nutritional deficiencies will contribute to the differences seen in young and old subjects with respect to their immune function tests. The only nutrition criterion included in the SENIEUR protocol is body mass index (BMI); therefore, the protocol does not allow for screening of other nutrition tests. There is vast literature

suggesting that specific nutrient deficiencies can also affect immune function (Beisel et al., 1981, Katka, 1984; Kuvibidila and Baliga, 2002; Lesourd, 1995; Meydani and Beharka, 1996; Shankar and Prasad, 1998). Therefore, studies on age-associated changes in key components of immune function, innate and acquired, with different subject selection schemes will be reviewed here.

1.3 Skin and mucous membranes

The skin and mucous membranes are the body's first natural defense against foreign microbes. With ageing, there are changes in the skin such as drying, thinning, and decreased blood flow. The mucous membranes also become dry because of decreased protein synthesis and keratinization, and therefore can become more susceptible to injury. The changes in the integrity of the mucous membranes may help to promote bacterial invasion. Changes in extracellular matrix proteins such as fibronectin and vitronectin, which are important for bacterial adherence, have been associated with immobility and frailty (Bell and High, 1997).

1.3.1 Natural killer cells

A small group of peripheral blood lymphocytes that fail to express membrane molecules that distinguish T- and B-lymphocytes are called null cells. These cells also fail to display Ag-binding receptors of T or B cell lineage, and hence lack qualities of immunologic memory and specificity. One functional population of null cells called NK cells are large, granulated and constitute 5-10% of total peripheral blood lymphocytes in humans (Kuby, 1997). NK cells express CD16-56 membrane receptors, and play an important role as defenses against tumor and virus-infected cells in two ways. In the nonspecific, Ab-independent process, the NK cells make direct contact with the membrane of the tumor cell. Then, degranulation of perforin-containing granules results in formation of pores in the target cell due to released perforin, and finally lytic substances pass through these pores and bring about death of the target cell. NK cells also

mediate target-cell destruction by secretion of toxic molecules like tumor necrosis factor-alpha (TNF-α), which may initiate apoptosis. The Ab-dependent cell-mediated cytotoxicity (ADCC) involves NK cells that express CD16, a membrane receptor for end of an antibody molecule. These NK cells bind to antitumor antibodies and destroy the tumor. Other cells that exhibit ADCC are monocytes, macrophages, neutrophils, eosinophils etc (Kuby 1994).

The functions and activity of NK cells with aging has been studied extensively (Burns and Goodwin, 1997; Ginaldi et al., 1999). The results of these studies have yielded conflicting results, probably due to differences in methodologies and subject selection. Very few studies, primarily in animal models, have suggested an age-related decline in NK cells to perform cytotoxic activity such as lysing of spleen cells, or tumor cell lines like Daudi cells (Burns and Goodwin, 1997; Hsueh et al., 1996; Kutza and Murasko, 1994; Ogata et al., 1997). Most studies have found that the proportion or number of NK cells are increased in old individuals compared to young, irrespective of the marker used to characterize NK cells (Facchini et al., 1987; Krishnaraj and Blandford, 1988; Krishnaraj and Svanbourg, 1992; Krishnaraj, 1997; Sansoni et al., 1993; Tilden et al., 1986; Vitale et al., 1992). Few other studies have found no change between age groups on proportion or number of NK cells (Cakman et al., 1996; Krause et al., 1999; Ogata et al., 1997). With respect to NK cell function, most studies have suggested increases (Krishnaraj, 1997; Krishnaraj and Svanbourg, 1992; Lesourd and Meaume, 1994; Tilden et al., 1986) or no change (Facchini et al., 1987; Krause et al., 1999; Sansoni et al., 1993; Vitale et al., 1992) in tests of NK cell cytotoxicity with age.

For most part, the studies that have indicated an impairment in NK cell function with age, have been those which have found an increase in NK cell number in older individuals compared to young, but have failed to show a corresponding increase in NK cell cytotoxic activity (Facchini et al., 1987; Sansoni et al., 1993; Vitale et al., 1992). The investigators suggest that even though increases in NK cell number are noted, NK cell function on a per-cell basis declines with age,

therefore resulting in similar levels of absolute NK cell activity in all age groups. In a study by Ogata et al. (1997), an age-associated decline in NK cell activity was seen on a single-cell basis. However, unlike the previous studies mentioned, the proportion and numbers of NK cells did not have a significant correlation with age, suggesting that NK activity was indeed reduced with age. In contrast, some studies have observed an increase in NK cell function with age (Krishnaraj and Blandford, 1988; Krishnaraj, 1997; Tilden et al., 1986). Tilden and colleagues (1986) found that an age-associated increase in circulating NK cells in apparently healthy men and women were accompanied by an increase in the cytotoxic activity of NK cells against K-562 tumor cells.

The literature on NK cells with age presents mixed findings. It is interesting to note that many of the researchers who observed an increase in NK cell function did not use the SENIEUR protocol (Krishnaraj, 1997; Tilden et al., 1986), and conversely age-related declines in NK activity was seen in studies that fulfilled the SENIEUR selection criteria (Ogata et al., 1997; Vitale et al., 1992). Regardless of findings from different studies, the general trend is that NK cell numbers increase with age and NK cell function is preserved or enhanced, and therefore innate immunity does not undergo significant declines with age (Ginaldi et al., 1999). The mixed findings on NK cells and their activity with age may be related to other factors besides health status of subjects namely nutritional status.

1.3.2 Phagocytic cells: Monocytes/Macrophages and Neutrophils

The chief phagocytic cells that are an important component of non-specific immunity are macrophages and neutrophils. Both cells types, although different in their structures, are formed from granulocyte-monocyte progenitor cells in the bone marrow during hematopoiesis and have similar phagocytic mechanisms. Monocytes and macrophages represent the mononuclear phagocytic system. Granulocyte-monocyte progenitor cells in the bone marrow differentiate into promonocytes, which enter blood and differentiate into monocytes. Monocytes then circulate in

blood for about 8 hours, during which time they enlarge and migrate into tissues and again differentiate into tissue macrophages. Neutrophils are granulocytic cells (stains acid and basic dyes) produced in the bone marrow during hematopoiesis, and are often referred to as polymorphonuclear leukocytes (PMNs) due to their multi-lobed nucleus (Kuby, 1997).

Phagocytes can ingest and digest antigens like whole microorganisms, insoluble particles, injured and dead host cells, cellular debris and activated clotting factors. The first step of phagocytosis is called chemotaxis, and involves the attraction and movement of phagocytes towards antigenic substances, in response to cytokines. This movement then leads to adherence of the Ag to the phagocytic cell membrane. Adherence then leads to an extension of membrane around the attached material, by protrusions called pseudopodia. The phagocyte then completes the engulfing of the foreign material and encloses it within a membrane-bound lysosome to form a phagolysosome. The engulfed material is then killed by two ways, namely oxygen-dependant and oxygen-independent mechanisms. Oxygen-dependant killing mechanisms involve the generation of toxic oxygen-derived radicals (superoxide, hydrogen peroxide, and hydroxyl radical) because of oxidative burst. Oxygen-independent killing mechanisms involve degradation activities by lysozyme, hydrolytic enzymes and cytotoxic peptides called defensins in the absence of oxygen. Finally, the digested or degraded material of the phagolysosome is eliminated by exocytosis. The phagocytic rate can be increased in the presence of substances called opsonins (e.g. antibody and complement), which can bind to antigen and phagocytic cells (Kuby, 1997).

Various tests are routinely performed to determine function of phagocytic cells. They include enumeration of PMNs and monocytes; functional and metabolic tests concerning locomotional properties such as PMN random locomotion, spontaneous migration and chemotaxis, neutrophils shape, adherence to nylon fibers, actin polymerization etc.; and tests concerning phagocytosis and killing activities such as phagocytosis, respiratory burst and reactive oxygen species (ROS) generation, nitroblue tetrazolium (NBT) dye reduction, ADCC, superoxide

dismutase and myeloperoxidase activity of PMNs, and killing activity to organisms such as *Candida*, *S. faecalis* etc. Studies examining age-associated changes in phagocytosis and bactericidal capacity of PMNs and macrophages have yielded conflicting results. Most studies have shown a decline in phagocytosis with age (Emanuelli et al., 1986; Polignano et al., 1994; Rabatic et al., 1988) or no differences in phagocytic function (Corberand et al., 1986; Fietta et al., 1994; Niwa et al., 1989; Oyeyinka et al., 1995) among young and old subjects. In addition, with respect to number or proportion of phagocytic cells, most studies show no age-related declines in monocytes or PMNs (Fietta et al., 1994; Matour et al., 1989; Nijhuis et al., 1994; Oyeyinka et al., 1995). However, chemotactic response seems to be significantly impaired (Brosche and Platt, 1995; Corberand et al., 1981; Fietta et al., 1994; Niwa et al., 1989; Polignano et al., 1994), and neutrophils adherence tends to be normal or increased in healthy older adults (Corberand et al., 1981; Corberand et al., 1986; Ginaldi et al., 1999).

It is interesting to note that most of the studies reviewed here, particularly those that report an age-associated decline in phagocytosis and bactericidal function of phagocytes, did not employ the SENIEUR protocol in the subject selection (Emanuelli et al., 1986; Polignano et al., 1994; Rabatic et al., 1988). In contrast, most of the studies that report no differences in phagocytosis with age have used the SENIEUR protocol (Corberand et al., 1986; Fietta et al., 1994; Oyeyinka et al., 1995). A possible explanation to account for these differences may be the confounding factors such as poor health and inflammation status that may have contributed to the age-related declines in phagocytosis. In addition to the lack of well-defined subject selection criteria, it is possible that differences in findings of various studies can also be in part due to factors such as laboratory methodology, specific nutrient deficiencies etc. Therefore, more studies using detailed study design and methodology with strict inclusion criteria for health and nutritional status may be useful to examine this aspect of innate immune function and aging.

1.3.3 Lymphocytes: B- and T lymphocytes

Lymphocytes are generated in the bone marrow during hematopoiesis and constitute 20-40% of the total white blood cells (WBCs). They leave the bone marrow, circulate in the blood and lymph, and reside in the various organs of the lymphatic system. Lymphocytes are important immune cells and show attributes of specificity, diversity, memory and self/nonself recognition. These cells have membrane receptors, which help them recognize foreign antigens. Based on cell membrane components and function, lymphocytes can be divided into three cell types, B cells, T cells and null cells. All these cell types are small, motile, nonphagocytic, and cannot be distinguished morphologically. The B and T cells constitute the majority of the lymphocyte population (Kuby, 1997).

B lymphocytes mature within the bone marrow, and leave the bone marrow with a unique antigen-binding receptor on the membrane. The B cell receptor is an antibody molecule, a membrane-bound glycoprotein or immunoglobulin, capable of binding to the antigen. During the first encounter with an antigen for which its membrane-bound antibody is specific, the naïve B cell begins to rapidly divide and differentiate into memory B cells and effector cells called plasma cells. Memory B cells have a longer life span than plasma cells and continue to express membrane-bound antibody with the same specificity as their parent cell. Plasma cells, in contrast, do not produce membrane-bound antibody, but produce antibody in a form that can be secreted and these are the major effector molecules of humoral immunity (Kuby, 1997).

T lymphocytes also arise in the bone marrow, but unlike B cells, they mature in the thymus. During maturation, the T cell expresses a unique antigen-binding receptor on its membrane, the T-cell receptor. The T-cell receptors are different from the membrane-bound antibodies on B cells in that they recognize only antigens in association with cell-membrane proteins known as the major histocompatability complex (MHC) complex. When a naïve T cell first encounters an antigen associated with an MHC molecule on a cell, the T cell proliferates and

differentiates into memory T cells and effector cells. There are two subpopulations of T cells, T helper (T_H) and T cytotoxic (T_C) cells, which can be distinguished by their display of membrane glycoproteins CD4 and CD8 respectively. In response to an antigen-MHC complex, T_H cells become activated. The activated T_H cells become effector cells by secreting various cytokines, which are important in activating B cells, T_C cells, macrophages and other cells important for mounting an immune response. In addition, with the help of T_H-derived cytokines, the T_C cells that recognize an antigen-MHC complex proliferate and differentiate into cytotoxic T lymphocytes (CTLs). The CTLs generally do not secrete cytokines and mainly exhibit cytotoxic function against virus-infected cells, tumor cells, foreign tissue grafts etc (Kuby, 1997).

The ability of stem cells to undergo clonal proliferation declines with age, as does the maturation of thymocytes in relation to thymic involution (Chandra, 2002; Lesourd and Mazari, 1999). A major loss of thymic tissue occurs after sexual maturity; the thymic tissue is progressively replaced by fat with increasing age and new T lymphocyte generation is almost absent in older individuals (Lesourd and Mazari, 1999; Makinodan, 1995). Since the thymus is the site for T cell maturation, a decline in T cell phenotypic profile and function is expected, and this decline is closely associated with thymic involution. In fact, dramatic age-related changes are seen in T cells and their subsets. Here we will review some of the changes in lymphocytes, specifically T lymphocytes and their subsets with age.

Enumeration of lymphocytes and their subsets is important in evaluation of immune function. Knowledge of lymphocyte subsets numbers helps to determine the relationships between immune functions and the number and/or percent of cells responsible for these functions (Bogden and Louria, 1997). Circulating lymphocytes decrease with age (Chandra, 2002; Facchini et al., 1987; Lesourd and Mazari, 1999; Wikby et al., 1994), but this is not seen in the healthy older adults (Brosche and Platt, 1995; Mazari and Lesourd, 1998). For total T cells, a decline has been observed with age (Wikby et al., 1994; Hallgren et al., 1988), even in studies,

which have used the SENIEUR protocol (Facchini et al., 1987; Sansoni et al., 1993; Vitale et al., 1992). Some researchers, who have focused on mature versus immature T cells, have found that aged individuals express fewer mature CD3+ and more immature CD2+ CD3- cells, which is again reflective of thymic maturation and involution (Lesourd and Mazari, 1999).

With respect to subsets of T cells, certain subsets are more susceptible to decline with age than others. Most studies show no change with age in proportions or numbers of T helper (CD4+) cells (Facchini et al., 1987; Bell and High 1997; Krause et el., 1999; Lesourd and Mazari, 1999; Nijhuis et al., 1994; Oyeyinka et al., 1995; Vitale et al., 1992; Wikby et al., 1994). However, an interesting change in CD4+ lymphocytes is a shift from naïve cell subpopulations (CD45RA+) to those associated with activated or memory cells (CD45RO+) (Cakman et al., 1996; Cossariza et al., 1992; Nijhuis et al., 1994). For age-related changes in CD8+ cytotoxic cells, various studies have reported them as normal (Facchini et al., 1987; Hallgren et al., 1988; Krause et al., 1999; Oyeyinka et al., 1995), decreased (Sansoni et al., 1993; Vitale et al., 1992; Wikby et al., 1994) or increased (Bogden and Louria, 1997; Burns and Goodwin, 1997; Tilden et al., 1986).

The number of B cells shows very little changes compared to T cells (Bell and High 1997). In older adults, changes in B cell subsets show slightly more CD5+ (secrete autoantibodies) lymphocytes and decrease in CD5- (secrete antibodies against foreign antigens) lymphocytes. Overall, number or proportion of B cells shows little (Cobleigh et al., 1980; Sansoni et al., 1993; Wikby et al., 1994) or no change (Becker et al., 1979; Cakman et al., 1996; Facchini et al., 1987; Nijhuis et al., 1994; Oyeyinka et al., 1995) with age. If lymphocyte numbers are altered with age, then their functions as well as interactions with one another and other cells may be affected, leading to marked changes in immune response. The next section reviews some of the major functional changes of these lymphocytes with advanced age.

1.3.4 Lymphocyte functions

Evidence from considerable number of studied suggests impairment in many lymphocyte functions with age, particularly T cell functions (Bogden and Louria, 1997; Burns and Goodwin, 1997). The most prominent change in immune function with aging is the decline in T cell proliferation (Bell and High 1997; Gillis et al., 1981; Murasko et al., 1987; Schwab et al., 1990; Wikby et al., 1994). This observation has been one of the earliest qualitative changes with age to be reported (Burns and Goodwin, 1997).

The T cell proliferation response is one of the most widely studied functional tests of immune function and is thought to be the *in vitro* correlate of the DTH skin test, as it mimics the in vivo T cell clonal expansion that occurs in the beginning of an immune response (Burns and Goodwin, 1997). The process of T cell proliferation starts when the cell binds to an Ag (processed by an accessory cell) or a mitogen, and the whole process results from complex interactions between the T cells, macrophages and accessory cells. Mitogens are proteins, some from plant sources, which can induce cell division in a large number of T- and B cells. Mitogens and antigens are different in that mitogens activate lymphocytes regardless of antigen specificity of the lymphocytes, and are therefore polyclonal activators, whereas, antigens activate only those lymphocytes which have surface receptors specific for each antigen (Kuby, 1997). The binding of the T cell to the mitogen results in the activation of key substances in the cell such as phospholipase C and protein kinases. The activation of protein kinases leads to increased transcription and translation of the gene coding for interleukin (IL)-2 (T cell growth factor or CD25+) and receptors for IL-2. IL-2 is therefore, autocrine and paracrine in function, i.e. the T cell is its producer and its target cell. When T cells bearing IL-2 receptor (IL-2R) are exposed to IL-2, they proliferate and give rise to daughter T cells. Accessory cells such as monocytes help T cells produce and respond to IL-2 by presenting Ag that occupies and cross-links T cell receptors.

In addition, accessory cells secrete IL-1 and other monokines that provide important additional signals for completing the activation of T cells (Burns and Goodwin, 1997; Kuby, 1997).

The reason for the decline in T cell proliferation that occurs with age has been suggested by various studies. An important contributor to hyporesponsiveness to mitogens is the reduction in number of cells that are responsive to the mitogens (Burns and Goodwin, 1997) and changes in T cell subsets (Lesourd and Mazari, 1999). An increase in memory T cells, which are poor IL-2 secretors, may also be another reason (Lesourd and Mazari, 1999). In addition, immature CD2+ CD3- cells, which may be present in more numbers, have lower capacity to proliferate (Lesourd and Meaume, 1994). Decreased lymphocyte reactivity is also at least partly related to age-related increases in membrane viscosity, which induces defects in signal transduction (Huber et al., 1991; Lesourd and Mazari, 1999). Contradictory to all of the above findings of decreased proliferation of T cells with age, a few studies that have employed the SENIEUR protocol indicate that lymphocyte or T cell proliferation is comparable in young and old subjects (Fagiolo et al., 1993; Krause et al., 1999; Lesourd and Mazari, 1998; Nijhuis et al., 1994). These findings suggest a possibility of bias in subject selection, as poor health and nutritional status or different genetic background may contribute to apparent age-related declines in the proliferation response.

Studies from several investigators, in humans and animals, have demonstrated that secretion of IL-2 by cultured T cells is decreased in older adults, and subsequently the reduced IL-2 produced from these T cells limited T cell proliferation (Caruso et al., 1996; Nagel et al., 1988; Song et al., 1993; Wu et al., 1986). However, even exogenous IL-2 did not restore the T cell proliferation from cells of older subjects (Gillis et al., 1981; Song et al., 1993). These findings imply that low IL-2 production may exist in conjunction with defect in IL-2R expression and/or function. Many studies have examined IL-2R expression. The cells from old donors had decreased cell membrane IL-2R expression, in addition to decreased IL-2 mRNA expression (Nagel et al., 1988; Song et al., 1993; Schwab et al., 1990). The secretion of soluble IL-2R (sIL-

2R) by stimulated cells of older subjects was also decreased (Candore et al., 1992; Caruso et al., 1996; Gillis et al., 1981). Decreased IL-2R expression on cells in conjunction with low amounts of cells capable of proliferation is thought to be the key factor for the overall impairment of the T cell proliferation response in the old compared to young individuals. Similar to proliferation results, contradictory findings have been reported in a few studies, where IL-2 secretion was comparable in young and old persons; these findings are in studies where SENIEUR protocol was used (Ahluwalia et al., 2001; Myśliwska et al., 1998; Nijhuis et al., 1994). This again suggests the possibility of lack of well-defined subject selection, which may be caused by presence of infection, inflammation, poor health, nutritional deficiencies, etc. in the populations studied in previous studies.

Lymphocyte responses are regulated by cytokines. These are messenger molecules, which act as signals between immune and other cells (Kuby, 1997). Helper T lymphocytes can be divided into 2 distinct subsets, T_H1 and T_H2, based on their cytokine production (Kuby, 1997). The T_H1 pathway is associated with cell-mediated immunity and production of IL-2, IL-12 and interferon (IFN)-γ, and is inhibited by IL-4 and IL-10. This pathway favors expansion of CD8+ effector cells and macrophage activation, and is triggered during infections with intracellular bacteria, certain viruses and parasites (Boppana, 1996; Bell and High, 1997). In contrast, the T_H2 pathway is associated with Ab-responses and predominates during helminthic infestations. In this pathway, Ab-responses such as immunoglobulin (Ig) production, eosinophils differentiation and activation occurs due to effect of cytokines such as IL-4, IL-5, IL-6, IL-10, and tumor necrosis factor (TNF)-α (Boppana, 1996; Bell and High 1997). In humans, T_H1 and T_H2 cells are not only different in their cytokine production, but also in their responsiveness to cytokines. Further, T_H1 cells are cytolytic in function and provide B cell help for IgM, IgG and IgA (but not IgE) production whereas T_H2 cells are not cytolytic and induce IgM, IgG, IgA, and IgE production by B cells (Boppana, 1996).

Changes in T_H1 and T_H2 subsets of CD4+ cells due to altered cytokine production has been thought to be an important contributor to immune ageing and compromised immunity (Bell and High, 1997; Lesourd and Mazari, 1999; Rink et al., 1998). Specifically, reports from many studies indicate a shift from T_H1 to T_H2 responses, due to decreases in T_H1 cytokines such as IL-2 (Song et al., 1993) and IFN-γ (Cakman et al., 1996); and increases in T_H2 cytokines such as IL-4 (Nijhuis et al., 1994), IL-6 (Myśliwska et al., 1998) and TNF-α (Maes et al., 1999). This decrease in T_H1: T_H2 may be of importance for age-related immune changes, as T_H1 induces maturation and activation of cytotoxic T cells which decreases with age, and T_H2 increases B cell Ig production which increases with age (Lesourd and Mazari, 1999). It is interesting to note that very few studies have found that T_H1 (Ahluwalia et al., 2001; Fagiolo et al., 1993; Myśliwska et al., 1998) and T_H2 (Caruso et al., 1996; Peterson et al., 1994) cytokine production are not different between young and old individuals. Most of these studies have employed the SENIEUR protocol (Caruso et al., 1996; Fagiolo et al., 1993) and some have used additional criteria to ensure adequate nutritional status along with the SENIEUR protocol (Ahluwalia et al., 2001), and therefore have not found differences across age groups on cytokine production. This suggests that immune senescence is not merely a shift from T_H1 to T_H2 responses, and that overall balance of different cytokines and production of important cells may be brought about by factors related to health and nutritional status (Ahluwalia et al. 2001; Bell and High 1997; Lesourd and Mazari, 1999).

Functional changes in B cells with age have become more apparent recently, as studies in the past focused more on T cell functions, as T cells are thought to be more vulnerable to age-related declines (Bogden and Louria, 1997). For many antigens, B cells require helper factors generated by T cells, to produce antibodies. The Ab-responses of the B cells to such antigens (T cell dependant) are reduced in older adults, and the affinity of the Ab may be decreased (Chandra, 2002; Lesourd and Mazari, 1999). The decrease in T cell dependant Ab response is thought to be

associated with increased anti-idiotype antibodies (Lesourd and Mazari, 1999). Anti-idiotype antibodies production is the accumulation of antibodies directed against other antibodies, and this leads to production of antibodies with lower antigenic affinity. The lower affinity of the Ab produced has been linked to changes in B cells subsets, i.e. increase in CD5+ cells that secrete autoantibodies (Weksler, 1995). In addition, studies have also shown that the production of autoantibodies does increase with age (Hallgren et al., 1973).

1.3.5 Other macrophage functions: interactions with T cells

Cell-mediated immune responses are brought about by specific signals between cells of the immune system. One such important response occurs between T lymphocytes and their Ag-presenting partners, namely macrophages. Briefly, when some subpopulations of activated T_H cells encounter certain types of antigens, they secrete cytokines that induce a localized inflammatory reaction called the DTH response. This *in vivo* reaction is a secondary immune response characterized by large influxes of nonspecific inflammatory cells, and macrophages serve as principal effector cells of the reaction (Bell and High 1997; Kuby, 1997).

Macrophages are major participants in the DTH response as they gather foreign particles and present them to the T lymphocytes. In the process, they can secrete either IL-1, which promotes T cell activation, or prostaglandin-E2 (PGE-2), which inhibits T cell activation. Therefore, macrophages play an important role in regulating T cell activation (Bell and High 1997; Kuby 1994). Studies have shown that the T cell responses to Ag presentation by macrophages may be altered in older adults because of PGE-2 (Bell and High 1997). As a result, this impaired interaction between T cells and macrophages may bring about depressed DTH responses in older individuals. A study by Goodwin et al. (1982) supports the finding of decreased DTH response in older adults. In this study, 279 healthy older subjects from the NMAP cohort underwent skin tests to four common antigens. Seventy-six of the 279 (33%) older

subjects had depressed skin test responses compared to young subjects, and responses of the older subjects were similar to chronically ill older subjects. Similar findings were noted in another study, in which SENIEUR protocol was used (Fietta et al., 1994). In this case, DTH responses were measured after 146 subjects ranging from 25 to 100 years in age were tested with seven antigens. The results showed that subjects who were over 66 years displayed reduced frequency of DTH to all recall antigens, and anergy was more frequent (29%) in older subjects (66-100 age group) than in the younger subjects (9.4% in 25-45 age group and 5% in 46-65 age group).

Numerous cytokines, primarily of the T_H1 subsets, play a role in generating an effective DTH response (Kuby, 1997). Here we will focus on some of the important proinflammatory cytokines such as IL-1 and IL-6, which are macrophage-derived and have been implicated in the pathogenesis of age-related diseases.

Studies in mice show that IL-1 secretion from monocytes/macrophages is reduced with age (Inamizu et al., 1985). In contrast, human studies show (with or without the use of SENIEUR protocol) either an increase of IL-1 production with age (Fagiolo et al., 1993) or no significant difference between young and old on IL-1 production (Ahluwalia et al., 2001; Wei et al., 1992). IL-6 and TNF-α are two other cytokines, which have proinflammatory functions similar to IL-1. Together, these three cytokines have been implicated as key products of activated tissue macrophages at the onset of an acute inflammatory response (Kuby, 1990). IL-6 is different from IL-1 and TNF-α in that it does not induce IL-8 (helps in neutrophils adherence and chemotaxis) and PGE-2 (Kuby, 1997). However, IL-6 is induced by IL-1 and TNF-α, and therefore, it can be an important indicator of those two cytokines (Brosche and Platt, 1995).

Many gerontologists have focused on IL-6 as an important cytokine for immune ageing. Large amounts of IL-6 result in chronic inflammation. There is increasing speculation that IL-6 contributes to the pathogenesis of age-related illnesses such as osteoporosis, arthritis, Alzheimer's disease, lymphoma, etc (Ershler, 1993). Most studies in humans have found that IL-6 production

increases with age (Ershler, 1993; Wei et al., 1992; Young et al., 1999), indicating increased T_H2 responses, even when health of the older individuals was maintained (Fagiolo et al., 1993; Myśliwska et al., 1998). Other studies have failed to show any difference across age groups on IL-6 production (Ahluwalia et al., 2001; Caruso et al., 1996; Maes et al., 1999), and most of these studies have used SENIEUR protocol and ensured nutrient adequacy as confounding factors (Ahluwalia et al., 2001; Caruso et al., 1996). Due to these conflicting findings in the literature related to the study design and subject characteristics, future studies are needed to examine the effect of increasing age on cytokine production.

1.4 Nutrition and immune function

Nutrition and immune function are interrelated. Epidemiological and clinical studies provide evidence that many nutrient deficiencies result in altered immunocompetence and increased risk of developing infections. The early work on nutrition and immune function was primarily based on findings from studies in nutritional deficiencies in young children from developing countries. Much evidence today points out that nutrition is an important determinant of immune function across age groups worldwide. Chandra (1991) has suggested five important concepts for nutrition and immune function interactions: 1) alterations in immune responses occur early in the course of reduced micronutrient intake; 2) the extent of immunologic impairment depends on the type of nutrient involved, its interactions with other nutrients, severity of disease, presence of infection, and age of the subject; 3) immunologic abnormalities predicts the risk of infection and mortality; 4) for many micronutrients excessive intake is associated with impaired immune responses; and 5) tests of immune function are useful in assessing physiologic needs and safe upper and lower limits of intake of micronutrients. Immunologic dysfunctions may occur because of single nutrient deficiencies, such as vitamin A, iron and zinc, or because of multiple nutrient deficiencies in conjunction with malnutrition (Beisel et al., 1981). Although several macronutrients, fat- and water-soluble vitamins, play a role in immune function, the following

section will focus on key nutrients that affect immune function and/or that are important for older adults, as they may have suboptimal status of these nutrients or these nutrients may be lacking in their diet.

1.4.1 Protein-energy malnutrition (PEM)

PEM can have detrimental effects on the immune system. Epidemiological studies, primarily with children, in developing countries have shown that PEM has been associated with morbidity and mortality due to presence of conditions such as upper respiratory tract infections and diarrhea (Chandra, 1991). Clinical studies and observations, in humans and animals, show that PEM contributes to impairment in nonspecific immune compartments such as complement, but the most affected are components of the cell-mediated immunity (Bell and High, 1997). Distinct changes are observed in the thymus and thymus-related functions. There is lymphoid atrophy especially in the liver and spleen, and a decline in serum thymic factor with PEM (Chandra, 1991). Studies from malnourished children also show that thymic changes contribute to a reduction in mature T cells, CD4+ cells, and CD8+ cells. As a result, important functions such as lymphocyte proliferation and DNA synthesis are reduced. In addition, DTH responses to recall and new antigens are reduced, and anergy may be prevalent (Chandra, 1991). Findings from co-culture experiments in malnourished and healthy children indicate a decrease in the Ab-producing cells and the amount of Igs secreted (Chandra, 1991).

Undernutrition is common among older populations. PEM affects many free-living older adults, but it is found largely in hospitalized older persons (Lesourd et al., 1998). PEM is also considered a major prognosis for mortality in older adults, as indicated in hospitalized patients (Sullivan et al., 1990). The impact of PEM on the immune response of older subjects is well illustrated in a study by Lesourd (1995). Two groups of older subjects were selected using the SENIEUR protocol, with one group being well-nourished and the other being undernourished

(with serum albumin between 30-35 g/L in the absence of acute phase reactions in the body). The third group comprised of younger subjects also selected using SENIEUR criteria. Although both groups of older subjects had lower CD3+ and CD8+ cells than the younger subjects, the undernourished group had lower CD4+ cells compared to the other groups. When comparing the two older groups, the undernourished group had lower lymphocyte proliferation response, DTH response, IL-2 production and Ab response to influenza vaccine.

Many studies confirm the negative effects of PEM on immunity across age groups. It is important to note, however, that most of the times isolated PEM is uncommon. PEM usually may be accompanied by infection and/or micronutrient deficiencies (Chandra, 1991). Therefore, the possible contributions of these factors on immune function must be noted while reviewing studies.

1.4.2 Vitamin A

Vitamin A plays an important role in maintaining the integrity of the mucosal epithelium. It has been called the 'anti-infective' vitamin; several epidemiological studies have found an association between severe vitamin A deficiency and increase in infections (Chandra, 1991). Vitamin A is important for gene expression, and therefore helps in cell proliferation and differentiation. All these functions make vitamin A important for immune function reactions. The effect of vitamin A on immune function has profound implications, especially for vulnerable populations, such as children and women, in developing countries where vitamin A deficiency is quite prevalent (Bhaskaram, 2002).

Animal and human studies have shown that vitamin A deficiency affects various immune function outcomes (Beisel et al., 1981). Vitamin A deficiency results in decreased lymphocyte proliferation, DTH responses, and numbers of total T cells, CD4+ and CD8+ cells (Beisel et al., 1981; Bhaskaram, 2002). Humoral responses are also affected with a decrease in Ab response to

antigens such as tetanus (Semba, 1992). High levels of vitamin A have been implicated in enhancing phagocytic function of neutrophils, IL-1 production of macrophages, and reducing severity and morbidity of infections (Bhaskaram, 2002).

1.4.3 Vitamin D

Vitamin D is an important immunomodulatory hormone. The active form of vitamin D, 1,25-dihydroxycholecalciferol is thought to interact with specific vitamin D receptors on immune cells such as monocytes and activated lymphocytes (Lemire, 1992). Vitamin D suppresses lymphocyte proliferation and Ig production in a dose-dependant manner, as it is a potent inhibitor of the TH_1 response and the TH_1 cytokines, IL-2, IL-12 and IFN-γ (Bell and High, 1997; Rigby et al., 1987). TH_2 cytokines remain unaffected by vitamin D (Bell and High, 1997). Vitamin D is also inhibits generation of cytotoxic and NK cells, whereas it enhances T suppressor cell activity. Expression of class II MHC molecules of lymphocytes and monocytes is also affected by vitamin D (Lemire, 1992). Vitamin D is important for the older population. Vitamin D deficiency may be common among some segments of the older adult population such as those who are institutionalized or homebound (Keane et al., 1995).

1.4.4 Vitamin E

Vitamin E is recognized as an important antioxidant and immune-enhancing vitamin. Along with other antioxidant vitamins, it is an important component of cell membranes; especially in immune cells as their high polyunsaturated fatty acid (PUFA) content may be a risk for oxidative damage. Vitamin E is helpful in protecting from harmful damage (Meydani and Beharka, 1996). In animals, experimental studies show that vitamin E reduces normal immune responses. Vitamin E deficiency results in impaired T and B cells functions such as lymphocyte proliferation (Corwin and Gordon, 1982), in addition to decreased neutrophil and macrophage

function of chemotaxis and phagocytosis (Harris et al., 1980). In humans, vitamin E deficiency is rare, and occurs along with conditions such as malabsorption, or HIV-infection (Meydani and Beharka, 1996). Kowdley et al. (1992) reported that a patient with vitamin E deficiency, due to malabsorption, had depressed T cell responses to PHA and Con A, DTH responses and IL-2 production. In premature neonates who demonstrate some level of this vitamin deficiency, Chirico and colleagues (1983) found impaired neutrophil function with reduced phagocytosis, bactericidal capacity and chemotaxis. In both studies, the T cell and neutrophil functions were improved when vitamin E supplementation was provided.

Some studies involving vitamin E supplementation in normal laboratory animals and humans have suggested that vitamin E intake in excess of recommended amounts have immune-enhancing effects (Meydani and Beharka, 1996). Moriguchi and colleagues (1990) found that supplementation of rats for 10 days with 100-2500 ppm vitamin E resulted in increased mitogenic response of splenocytes, NK cell cytotoxicity and phagocytic function of macrophages. The findings of a double-blind randomized study by Meydani et al. (1997) in healthy older adults > 65 years, supplemented with placebo, 60mg/day, 200 mg/day or 800 mg/day of α-tocopherol for 235 days, showed that groups consuming 200 or 800 mg/day vitamin E had increase in Ab response to hepatitis B. In addition, all vitamin E supplemented groups had enhanced DTH responses and 30% lower incidence of self-reported infections. In contrast, a recent study by Gratt et al. (2002) reported different findings from Meydani’s study on the incidence of infections with vitamin E supplementation. Six hundred and fifty two non-institutionalized older individuals were divided into four groups; each group was given either multivitamin-mineral supplement (physiologic dose) or vitamin E (200mg) or multivitamin-mineral plus vitamin E supplement or placebo for 15 months. The findings showed that neither vitamin E nor multivitamin-mineral supplementation showed a favorable effect on the incidence and severity of acute respiratory tract infections.

Further, for subjects given vitamin E, severity of illness was worse than those who were not given vitamin E.

1.4.5 Vitamin C

Vitamin C affects immunity as it plays an important role in phagocytic functions of neutrophils and macrophages. It is important for the synthesis and assembly of microtubules of phagocytes. Large concentrations of vitamin C are normally present in phagocytic cells (Myrvik, 1999). The amount of vitamin C in WBCs may decrease during viral infections, pregnancy and in older persons (Beisel et al., 1981). Animal studies have shown that vitamin C deficiency reduces DTH responses to skin test antigens (Myrvik, 1999). This is caused by an inability to mount a local inflammatory response rather than a defect of lymphocytes to recognize or process Ag (Beisel et al., 1981). Studies also show that vitamin C deficiency does not affect levels of antibodies (Chandra, 1991). However, cytotoxic activities of lymphoid cells are impaired, even if number of T cells is normal (Myrvik, 1999).

Some cross-sectional studies have shown that vitamin C status, measured by leukocyte and plasma ascorbate levels, is lower in older than in young adults, especially institutionalized older adults (Burr et al., 1974; Cheng et al., 1985). In contrast, some other cross-sectional and longitudinal studies have found that in apparently healthy, well-nourished older persons in the United States, the incidence of vitamin C deficiency in the old is not greater compared to young adults and that there is no decrease in plasma ascorbate with aging (Garry et al., 1982, 1987; Jacob et al., 1988). Therefore, decreased vitamin C status, if present, as in institutionalized older individuals and those with inflammation and infection (Cheng et al., 1985), may have important implications for decreased immune function.

1.4.6 Vitamin B_6

Vitamin B_6 or pyridoxine is an important coenzyme for many metabolic reactions such as protein and nucleic acid synthesis, transformation of amino acids by decarboxylation, deamination and transamination etc. (Bell and High, 1997; Myrvik, 1999). Pyridoxine deficiency leads to impaired DNA and protein synthesis along with impaired cell-mediated and humoral immune functions (Beisel et al., 1981). In vitamin B_6 deficient animals, atrophy of lymphoid tissue results in smaller thymus and decreased thymic hormone activity (Bell and High, 1997; Chandra, 1991). Lymphocyte function is decreased due to impaired nucleic acid synthesis (Bell and High, 1997). This results in reduced T cell cytotoxicity, depressed DTH reactions, delayed rejection of allografts, and decreased lymphocyte proliferation response to mitogens and antigens. In addition to lymphocyte function, Ab formation and Ab response to vaccines are reduced (Sudhakaran and Chandra, 1990)

Isolated vitamin B_6 deficiency in humans is rare (Chandra, 1991). It exists along with other micronutrient deficiencies. Vitamin B_6 deficiency can be found in free-living older adults and is quite common in institutionalized older individuals (Lesourd et al., 1998). In the NMAP cohort, approximately one-third of the older subjects were moderately vitamin B_6 deficient, as indicated by plasma PLP levels of below 30 nmol/L (Garry et al., 1982). The prevalence of vitamin B_6 deficiency may have important implications for the immune function of the older persons. In a study by Talbott et al. (1987), the effect of pyridoxine supplementation on lymphocyte responsiveness was investigated in persons aged 65-81 years. Subjects received 50 mg/day pyridoxine or placebo. After one and two months of supplementation, increases were seen in plasma PLP levels, lymphocyte proliferation to PHA, PWM and *S. aureus*, lymphocyte blastogenesis to Con A, and percentages of CD3+ and CD4+ cells. This suggests that improving vitamin B_6 status is important in stimulating immunocompetence in B_6 deficient older adults.

1.4.7 Vitamin B_{12}

Vitamin B_{12} has important functions in the body. It is needed for production of RBCs and myelin sheath covering nerve tissue. In another especially important function, vitamin B_{12} is needed for cell growth and division, as it serves as a coenzyme for DNA/nucleic acid synthesis (Myrvik, 1999). Due to its hematological functions, deficiency of vitamin B_{12} results in megaloblastic anemia, as well as decreased folate use and transport thereby affecting folate metabolism. Its important function in DNA synthesis has implications for maintaining immune functions, such as proliferation and responsiveness of lymphocytes (Myrvik, 1999).

Observations on the effect of vitamin B_{12} deficiency on immune function outcomes, comes from studies with patients or subjects with vitamin B_{12} deficiency or primary pernicious anemia, as vitamin B_{12} deficiency cannot be induced in animals (Myrvik, 1999). MacCuish et al. (1974) found that although T and B cells were normal in the peripheral blood of patients with pernicious anemia, their lymphocyte responses to PHA were significantly depressed. Neutrophil function is also impaired in patients with pernicious anemia, as indicated by impaired ability to perform phagocytosis and bactericidal function on *S. aureus* (Kaplan and Basford, 1976). Katka (1984) investigated the functions of lymphocytes in 23 patients with pernicious anemia. E-rosette forming cells and surface Ig positive lymphocytes in peripheral blood, PHA responses of peripheral blood lymphocytes and tuberculin skin test were studied before and during specific treatment with vitamin B_{12}. The results of the *in vitro* tests showed no disturbances in the functions of lymphocytes before or during treatment. However, the tuberculin skin test changed from negative to positive in 5 out of 7 cases tested. This indicates that the improvement in vitamin B_{12} status was associated with significantly improved DTH function. Humoral immunity may also be affected by vitamin B_{12} deficiency (Fata et al., 1996). For older adults, this important function of vitamin B_{12} in immune reactions may be significant, because vitamin B_{12} deficiency is more common in older adults because of decreased production of intrinsic factor (Bell and High,

1997). In a study of 152 geriatric outpatients, 14.5% of the subjects were considered to have vitamin B_{12} deficiency, based on serum cobalamin level $\leq$ 300 pg/ml and levels of serum methylmalonic acid and/or homocysteine elevated to > 3 standard deviations (Pennypacker et al., 1992). The role of vitamin B_{12} status and immune function in older adults, however, has not been examined extensively.

1.4.8 Folic acid

Folic acid, like vitamin B_{12}, is required for cellular replication. In folate deficiency, synthesis of thymidylate is inhibited, resulting in megaloblastic changes in replicating cells such as RBCs and leukocytes. These megaloblastic changes are evident in few weeks of insufficient folate intake, as stores are depleted fairly rapidly. There is limited literature examining the relationship of folic acid and immune function. Folic acid deficiency depresses immune function in both animals and humans (Boles et al., 1982; Gross et al., 1975; Gross et al., 1980). Deficiency of folate can reduce T cell functions to PHA and T cell cytotoxic activity (Myrvik, 1999). In patients with megaloblastic anemia, DTH responses to skin test antigens are reduced (Gross et al., 1975). Phagocytosis of neutrophils is impaired in patients with decreased serum folate levels; the addition of folic acid to neutrophils restores the phagocytic function (Boles et al., 1982).

The aging process has not been associated with a reduction in the ability to utilize folate or decrease in serum folate or erythrocyte folate in some studies (Bailey et al., 1984; Rosenberg, 1992). In contrast, many reports indicate that homocysteine increases as a function of age (Selhub et al., 1993), and this increase may result from an age-related decline in important enzymes involved in homocysteine metabolism (Gartler et al., 1981). Therefore, inadequate folate status, which may be present in older adults, can decrease immune function.

1.4.9 Iron

Iron deficiency is the most common micronutrient deficiency in the world (Oppenheimer, 2001). Iron deficiency not only has important hematological implications, but also affects immune function. Iron is required for normal cellular enzyme function and DNA synthesis (Myrvik, 1999). Iron-deficiency affects both cell-mediated and humoral components of immunity; in addition, it is associated with increased incidence of infection and morbidity. Numerous observational and intervention studies have been conducted in iron-deficient children, adults and animals to examine the relationship of iron status with immune function (Kuvibidila and Baliga, 2002).

Many studies in iron-deficient children and adults, and laboratory animals have investigated tests of acquired immunity (Kuvibidila and Baliga, 2002). Cross-sectional studies comparing iron-deficient and iron-sufficient adults and children have demonstrated a decline in the proportion of T lymphocytes, although number of T cells decline or remain unchanged (Bagchi et al., 1980; Berger et al., 1992; Thibault et al., 1993). CD4+ and CD8+ cells also decline or remain normal in iron-deficient subjects (Berger et al., 1992; Thibault et al., 1993). Declines are seen with iron-deficiency in T cell functions including proliferation response to mitogens such as PHA and candida, and DTH responses (Macdougall et al., 1975; Swarup-Mitra and Sinha, 1984), and IL-2 production (Thibault et al., 1993). The number and proportion of B cells and Ig production, in humans, remain unchanged or slightly increased (Bagchi et al., 1980; Chandra and Saraya, 1975; Macdougall et al., 1975; Kuvibidila and Baliga, 2002).

Neutrophil functions have also been assessed in many studies in iron-deficient humans and animals (Kuvibidila and Baliga, 2002). One consistent finding is that in iron deficiency phagocytic and opsonic activity of neutrophils are normal (Chandra and Saraya, 1975; Yetgin et al., 1979), whereas bactericidal capacity of neutrophils is reduced (Macdougall et al., 1975; Walter et al., 1986). NBT dye reduction test and myeloperoxidase enzyme activity (iron-

dependant enzyme involved in neutrophil bactericidal killing) have been reported to be normal (Macdougall et al., 1975; Yetgin et al., 1979) or decreased (Chandra and Saraya, 1975) with iron-deficiency. Furthermore, the neutrophils functions of killing or enzyme activity that decrease with deficiency are restored after iron supplementation (Kuvibidila and Baliga, 2002; Walter et al., 1986). With respect to macrophages, studies have shown that macrophage migration inhibitory factor is reduced (Swarup-Mitra and Sinha, 1984) whereas IL-1 production (Bhaskaram, 2002) and Ab-dependent cell-mediated cytotoxicity (Bagchi et al., 1980) are not reduced in iron-deficient humans.

Iron overload has been less studied than iron deficiency, mainly because it is not as prevalent as iron deficiency. Nevertheless, iron excess can also impair immune functions. Iron overload can impair T cells, B cells and neutrophils, in ways similar to the studies mentioned above with iron deficiency (Kuvibidila and Baliga, 2002).

Studies on the effect of iron deficiency on infection in humans show conflicting findings. Some studies demonstrate that iron promotes infection, and therefore iron-deficiency is beneficial in protecting against infections (Brusner et al., 1993; Kuvibidila and Baliga, 2002). In contrast, other studies have found that iron protects against infection, and therefore, iron-deficiency promotes infection (Berger et al., 1992; Kuvibidila and Baliga, 2002). Further, other studies have suggested that iron alone may not determine the susceptibility to infection (Heresi et al., 1995; Idjradinata et al., 1994). In summary, studies with iron supplementation have shown that iron supplementation may have beneficial (De Silva et al., 2003; Hussein et al., 1988) or adverse (Barry and Reeve, 1977; Brusner et al., 1993) effects or that iron may not alone determine susceptibility to infection and illness (Heresi et al., 1995; Menendez et al., 1997). The conflicting findings on the relationship of iron status and infection may be related to the means of iron intervention (parenteral iron or oral iron supplement or food fortification), dose of iron supplements, presence of underlying concurrent nutrient deficiencies and inflammation, age and

iron status of study participants. In conclusion, the literature on this topic is controversial although recent meta-analyses show that iron supplementation is not associated with increased risk of infections (Oppenheimer, 2001).

1.4.10 Zinc

Zinc plays an important role in the immune response. It is required as a cofactor for at least 90 metalloenzymes, and many of these enzymes are important for transcription and translation. Because cells of the immune system undergo continuous proliferation and differentiation, they require many zinc-dependant metalloenzymes. Therefore, zinc deficiency affects many immune function responses. This is illustrated by many numerous studies in animals and humans (Myrvik, 1999; Shankar and Prasad, 1998). Acquired zinc deficiency is a result of low intake from food and can coexist with other micronutrient deficiencies like PEM, whereas inherited zinc deficiency or acrodermatitis enteropathica is an inherited defect in the intestinal absorption of zinc.

Zinc is important for thymulin secretion and therefore, zinc deficiency results in decreased thymulin and cell-mediated immune functions in animals and humans (Bhaskaram, 2002; Shankar and Prasad, 1998). Zinc deficient children show lymphopenia, delayed wound healing, thymic atrophy, reduced capacity to show DTH responses, and increased susceptibility to infections. Further, zinc supplementation of children has been shown to improve CD3+ and CD4+ subsets, along with an increase in CD4+: CD8+ ratios (Sazawal et al., 1997). In zinc deficient animals, reduced numbers of T and B cells are observed in addition to reduced capacity of these lymphocyte to proliferate in response to mitogens (Fraker et al., 1986; Gross et al., 1979). Studies in humans and animals indicate that NK cell activity is also decreased in zinc deficiency (Allen et al., 1983; Fernandes et al., 1979). PMN function is also altered in zinc deficient animals and patients with acrodermatitis enteropathica. In most cases, absolute numbers

of PMN remain unchanged, and impaired chemotactic responses are reversed by addition of zinc (Shankar and Prasad, 1998; Weston et al., 1977).

Zinc deficiency has been shown to affect infection. Clinically, zinc deficient children show increased susceptibility to infections. Several investigators have found a significant association between low plasma zinc and respiratory tract infections (Bhandari et al., 1996; Bhaskaram, 2002). Administration of zinc supplements reduces infections in children (Sazawal et al., 1997); similar findings have been noted in animals (Shankar and Prasad, 1998).

Zinc deficiency is frequently reported in older adults (Prasad et al., 1993). Bogden and colleagues (1987) found that 90% of free-living older adults in their study did not meet the RDA of 15 mg/day. Therefore, it has been suggested that zinc deficiency may play an important role in age-related immune declines.

1.5 Nutrition, immune function and aging

A dysregulation or decline in immune function occurs with increasing age, particularly in cell-mediated immune functions (as reviewed above in section 2), and this may contribute to an increase in infections in older individuals. Nutrition plays an important role in maintaining immunocompetence in the older adults (High, 1999). Even though micronutrient deficiencies are rare in developed countries, the older population at increased risk, because of physiological changes associated with aging (Bell and High, 1997), or social and economic factors or because of being homebound or institutionalized (High, 1999). Many older persons do not meet the Recommended Dietary Allowances (RDA) for a number of nutrients including vitamins B_6, B_{12}, D and folate, as well as minerals calcium, magnesium and zinc (Tucker, 1995). Older adults have high prevalence of nutrient deficiencies (Chandra, 2002), due to variety of reasons; therefore, the declines in immune responses with aging may not be directly due to increased age, but due to poor nutritional status (Brosche and Platt, 1995). This is illustrated by studies comparing young

and older adults on immune function where adequacy of health and adequacy of nutritional status was ensured; the results of these studies show that most immune functions are preserved in older adults who are healthy and well nourished. This has primarily been shown by Lesourd's group (Lesourd, 1995; Lesourd and Meaume, 1994; Lesourd et al., 1998) and studies by Ahluwalia and coworkers (Ahluwalia et al., 2001; Krause et al., 1999). Another set of studies in older adults has focused on the effect of micronutrient (vitamin and mineral) deficiencies and the effects of correcting nutrient deficiencies on immune response. In other studies, researchers have also addressed the issue of whether micronutrient supplementation might improve immune functions even in the absence of deficiencies. Findings from some of these studies will be reviewed in this section.

1.5.1 Cross-sectional studies of single nutrients and immune function

Several clinical trials of single nutrients have been conducted with older subjects with or without deficiencies. Mazari and Lesourd (1998) compared groups of older adults selected by SENIEUR protocol, on immune function tests. SENIEURS were divided into two groups based on their nutritional status: Eighteen SENIEURS had low folate status, with serum folate < 5 μg/L, and 25 SENIEURS were healthy and had no evidence of any nutritional deficiencies. Although CD3+ cell numbers were comparable in both groups, there was a trend for lower CD3+ numbers in the low folate group. The low folate SENIEURS had significantly lower numbers of CD4+ and CD8+ cells than the healthy SENIEURS; the decreases in CD8+ correlated with decreases in CD3+. Increases in CD57 cells (NK cells) were noted in the SENIEURS with low folate compared to the other group, and this increase correlated with increase in CD2+ CD3- cells (immature T cells). Important T cells functions such as lymphocyte proliferation response to PHA and IL-2 production were lower in the low folate group versus the healthy group. The group with low folate also produced lower amounts of IL-6 than the healthy SENIEURS,

although this difference was not statistically significant. Although this study noted a correlation between folate status and immune responses, the effect of folate supplementation was not studied.

In another prospective study, fifteen older patients with low serum vitamin B_{12} were compared to fifteen matched healthy controls with normal serum vitamin B_{12} on serum antibody responses to the pneumococcal polysaccharide vaccine (Fata et al., 1996). Serum antibody titers to 12 pneumococcal serotypes were measured before and 4 weeks after vaccination. Individuals with low serum vitamin B_{12} had impaired Ab responses to the pneumococcal vaccine. The difference between the Ab titers before and after vaccination for all 12 serotypes was lower in the patients with low vitamin B_{12} levels than in the patients with normal levels. Similar to Mazari and Lesourd's study, this study did not investigate the effect of vitamin B_{12} supplementation on the two groups.

1.5.2 Supplementation studies with single nutrients and immune function

Other studies document the effect of single nutrient supplementation on immunity in persons who have low status for a particular nutrient. The effect of vitamin B_6 deficiency on immune response was studied in a depletion-repletion study with eight healthy old volunteers by Meydani et al. (1991). Subjects followed a 5-day baseline period; a vitamin B_6 depletion period of less than or equal to 20 days; three stages of vitamin B_6 repletion, each lasting 21 days; and a 4-d final phase. The amounts ingested during the different phases of the study were 3, 15, 22.5, and 33.75 μg/kg body weight/day, respectively. During the final phase, subjects ingested 50 mg vitamin B_6/day. Vitamin B_6 depletion significantly decreased percentage and total number of lymphocytes, mitogenic responses of peripheral blood lymphocytes to T- and B-cell mitogens, and IL-2 production. Immune test indices returned to baseline values after the third repletion period. The results suggest that the impairment of *in vitro* indices of cell-mediated immunity in healthy older adults by vitamin B_6 deficiency can be reversed by vitamin B_6 repletion. Although

Meydani's study showed improvements in immune function with adequate B_6, the study had a small sample size.

Some researchers investigated the effect of single nutrient supplementation on immunity in older individuals who were healthy and did not have any apparent nutrient deficiencies. Corridan and colleagues (2001) supplemented 52 healthy free-living older adults (> 65 years) with either placebo, or lycopene (13.3 mg/day), or β-carotene (8.2 mg/day) for 12 weeks. There was no significant difference in immune tests, namely WBC, lymphocyte and monocytes numbers; percentages of lymphocytes, monocytes, total T cells, T helper cells, T cytotoxic cells, and NK cells; or lymphocyte proliferation and IL-2 and IL-4 production in PHA-stimulated cultures, from baseline to after 12 weeks or across groups. Only lycopene and β-carotene improved in the groups that were supplemented compared to baseline. The authors suggest that low dose supplementation may not affect cell-mediated immune responses in a well-nourished older group, who may already possess optimal immune status. In another study, Meydani and colleagues (1990) studied the effect of vitamin E supplementation on cell-mediated immunity in 32 healthy older adults by a double-blind, placebo-controlled trial. Subjects received either placebo or vitamin E (800 mg dl-α-tocopheryl acetate) for 30 days. Vitamin E status and immune function tests were measured before and after supplementation. The vitamin E supplemented group had significantly higher α-tocopherol in plasma and PBMC's, elevated DTH responses, increased lymphocyte proliferation to Con A, increased IL-2 production, and decreased PGE-2 synthesis by PBMC's and plasma lipid peroxides compared to the other group. Their results indicate that even short-term vitamin E supplementation can improve cell-mediated immunity, which may be mediated by the decrease in PGE-2 and peroxidation products.

The effect of zinc on immunity of older individuals has also been documented in studies. Bogden et al (1990) determined the effect of one year of zinc supplementation on 63 healthy older adults aged 60-89 years. Subjects were given placebo, 15 mg zinc, or 100 mg zinc daily for

12 months in addition to a multivitamin/mineral supplement that contained no additional zinc. Zinc status and cell-mediated immune tests were measured at baseline, 3, 6, 12 and 16 months. Dietary intakes were also assessed at each visit. Intakes of folate, pyridoxine, α-tocopherol, copper, zinc, and magnesium were consistently below the RDA throughout the period of the study. For zinc status parameters, although plasma zinc increased in the 100 mg group, cellular zinc concentrations in erythrocytes, mononuclear cells, PMNs and platelets were not increased significantly with supplementation. NK cell activity remained unchanged in the placebo or 15 mg group, but was slightly enhanced with 100 mg/day supplementation. DTH responses and lymphocyte proliferative responses to PHA and PWM improved with supplementation, although the authors suggest that this is likely due to the one or more nutrients in the multivitamin/mineral supplement. It is interesting to note that the placebo group had significantly greater DTH response than that observed in any of the zinc treated groups. In another zinc supplementation study, Swanson et al. (1988) measured zinc status and visceral proteins such as albumin, transferrin etc of 53 healthy older subjects before and after 28 days of supplementation with 30 mg/day zinc. Of the 53 subjects, 19 were given no treatment, 17 were given placebo, and 17 were provided with zinc. Serum and urine zinc, and blood cell zinc increased with zinc treatment, whereas no change was observed in zinc content of platelets and leukocytes. In addition, no change was noted in concentrations of albumin, prealbumin, transferrin and retinol-binding protein or IgG, IgA, and IgM with zinc supplementation. The researchers conclude that aging may not necessarily be associated with impaired zinc status; however, the effect of zinc on immune function tests were not examined in that study.

1.5.3 Cross-sectional studies of multiple nutrients and immune function

In a study by Lesourd (1995), the influence of micronutrient status on lymphocyte proliferation was evaluated in four groups of subjects. Two of the groups were SENIEURS, one

with high folate and the other with low folate as determined by serum and RBC folate. The SENIEURS, by definition of SENIEUR protocol, had normal serum albumin, normal zinc, and no acute phase reactions. The third group comprised of JUNIEURS (healthy young adults), and the fourth group was healthy older adults with low albumin levels. Lymphocyte proliferation was evaluated by IL-2R kinetic appearance. No difference was noted between SENIEURS with adequate folate status and JUNIEURS; however a delayed IL-2R appearance was noted in healthy older adults with low albumin levels and SENIEURS with low folate. In another similar study by Lesourd's group (1995) the influence of undernutrition on Ab response was investigated. Two groups of healthy older adults, one with high albumin levels and the other with low albumin levels (between 30 and 35 g/L) were administered influenza vaccine. Although only 60-70% of vaccinated subjects increased their Ab responses, indicating that many people do not respond the first time, individuals with high serum albumin levels appeared to have normal responses but those with low albumin had very poor Ab responses. Both these studies indicate that the depressed immune function responses were related to undernutrition in the older groups studied.

Fülöp et al. (1999) investigated the relationship between influenza vaccine and nutritional status in a cohort of 23 institutionalized older subjects. About half of the subjects were not responsive to the vaccine. Nutritional parameters such as hemoglobin, hematocrit, total protein, iron, and vitamin E levels, as well as DHEA (an immunomodulatory hormone) and lymphocyte responses to Texas and Shangdong antigens were lower in the nonresponsive group compared to the responsive group. Similar to Lesourd's study, these findings suggest that in older adults, especially the frail, immune response is reduced with inadequate nutritional status.

The relationship between mega dose vitamin intake and immunologic function in the NMAP cohort was reported by Goodwin and Garry (1983). This was a cross-sectional study and not an intervention trial because the intent was not to attempt to supplement the group with micronutrients, rather to assess the relationship between the nutrient intakes and immune function

tests. Healthy older subjects were divided into groups based on total intake of nutrients (dietary + supplemental); subjects were in groups of top 25% or top 10% or bottom 75% of total intake for nutrients (Vitamins A, B_1, B_2, B_6, B_{12}, C, E, folate, niacin, and iron and zinc). No significant differences were noted in lymphocyte proliferation response to PHA, DTH responses, and manifestations of autoimmunity (autoantibodies and circulating immune complexes) between high intake and low intake groups for all nutrients. The only exception was vitamin C and iron, as the groups in the top 10% of intake for these nutrients had significantly fewer anergic subjects. Furthermore, some significant differences were noted for cell numbers in relation to intake of various micronutrients. Subjects in the top 25% of zinc intake had significantly higher mean absolute number of circulating blood neutrophils, and subjects with higher intakes of vitamins B_1, B_2, B_6, E, folate, and niacin had significantly decreased mean absolute lymphocyte numbers when compared to other groups.

Subsequently, Goodwin and Garry (1988) investigated the relationship between micronutrient deficiencies and depressed immunity in 230 older men and women of the NMAP group. Their results noted that although one-third of the subjects were anergic to DTH testing to four common antigens, there were no significant differences between the anergic and non-anergic group on nutrient intakes or blood levels. Spearman Rank correlations indicated no associations between blood levels of specific nutrients and immune function tests that were measured, namely DTH responses, lymphocyte and PMN absolute counts, lymphocyte proliferative responses to PHA, and levels of serum autoantibodies. Subjects in the bottom 5% or bottom 10% for various immune tests were compared with the remaining 90% of the group with respect to blood levels of specific nutrients. There were no differences between groups on levels of specific nutrients except for folate. Subjects in the bottom 5% of DTH scores and lymphocyte counts more frequently had low folic acid levels than subjects in the bottom 10% or top 90% group. The investigators suggested that subtle nutritional deficiencies might not be a noticeable contributor to

the immunodeficiency of aging. It is noteworthy that the NMAP cohort consisted of relatively affluent subjects, and use of prescription drugs were not considered as exclusion criteria.

In another cross-sectional study, Payette et al. (1990) examined the relationship between nutrition and immunologic status by regression analysis in 82 healthy free-living older adults. Anthropometric and dietary data, blood levels of eight nutrients, hematologic values and plasma fatty acids were included as the predictive variables; and cytotoxicity of NK cells and the activity of IL-2 were the immune variables studied. Based on regression analysis, none of the nutrition indicators (anthropometric, dietary or biochemical) was significantly correlated with cytotoxic activity of NK cells ($P<0.05$), and that the dietary intakes of vitamins E and D negatively influenced the activity of IL-2 ($P<0.007$).

1.5.4 Supplementation studies with multiple nutrients and immune function

Several groups have investigated the effect of multi-nutrient supplements on immune function of older adults. Some of these studies have determined the effect of multiple-nutrients on immunity of older adults at risk for deficiencies. In a large French study of 725 institutionalized older patients with varying health status, Girodon et al. (1999) determined the effect of trace element and/or vitamin supplements on DTH responses, humoral responses to influenza vaccine, and infectious morbidity and mortality. At baseline, the older patients were randomized into 4 groups: Subjects received either placebo (n=182), or trace element supplements with 20 mg of zinc and 100 μg of selenium (n=182), or vitamin supplements with 120 mg vitamin C, 6 mg β-carotene, and 15 mg α-tocopherol (n=180), or both trace elements and vitamins in the supplement (n=181). Baseline values for nutrients showed that prevalence of nutrient deficiencies was high and similar across the four groups. After six months of supplementation, the groups given trace element or vitamin supplement or both had an increase in nutrients in the serum, and the correction of nutrient deficiencies in the treated groups was

maintained in these groups. In contrast, the placebo group showed no change in serum values of nutrients. None of the treatments had any effect on DTH responses. Ab titers after influenza vaccine were higher in the trace elements group and vitamin and trace elements group, compared to the vitamin or placebo treated group ($P<0.05$). The number of patients without respiratory tract infections was higher in the trace elements treated group ($P=0.06$). None of the supplementation treatments reduced the incidence of urogenital infections. After two years, 519 patients were alive, and survival analysis showed no difference across the four groups. The most common cause of death in the non-survivors was cardiovascular diseases. Therefore, in this cohort of institutionalized older individuals, low-dose zinc and selenium supplementation was beneficial for the humoral response and reducing morbidity against respiratory tract infections at 6 month of supplementation.

In another European study, Buzina-Suboticanec et al. (1998) investigated the effect of vitamin supplements on DTH responses, to seven antigens, in a group of 70 ambulatory older subjects who had a high prevalence of nutrient deficiencies. Forty-two subjects were given a multi-vitamin supplement (B vitamins, vitamin A, C and E) whereas 28 subjects were given placebo daily for 10 weeks. At baseline, the prevalence of biochemical deficiency was high, especially for vitamin C, pyridoxine, riboflavin, iron and zinc, and males were more affected than females. A significant decline was noted in DTH with age as determined by correlation analysis ($P<0.01$). Vitamin supplementation for 10 weeks significantly improved the biochemical parameters of the supplemented vitamin. In addition, the vitamin supplementation had a positive and statistically significant effect on cutaneous reaction ($P=0.01$), vitamin-supplemented group, whereas no statistically significant changes in DTH were noted in the placebo group.

Studies have also investigated multi-nutrient supplementation and immunity in healthy older adults. Chandra (1992) supplemented 96 healthy older subjects (> 65 years) daily for a year with either placebo (n=48) or with a micronutrient formulation containing relatively low doses of

nine vitamins, five trace elements and higher levels of antioxidants vitamins C, E, and β-carotene (n=48), in a double-blind placebo-controlled fashion. Prevalence of nutrient deficiencies did not differ between the two groups at baseline. With supplementation, statistically significant reduction in deficiencies of vitamin A, β-carotene, vitamin B_6, vitamin C, iron and zinc were noted. Absolute amounts of lymphocytes or neutrophils did not change over the course of the study. However, many immunological tests improved with supplementation, including number of T and NK cells, lymphocyte proliferation to PHA, IL-2 production, IL-2R release, NK cell activity, and Ab response to influenza vaccine. Improvement in immunological responses was greater among subjects who had baseline nutrient deficiency, which was corrected with supplements. Infection-related illnesses were less frequent in the supplemented group versus placebo. The supplemented subjects experienced fewer days of illness per year because of infectious disease than the placebo group. These results suggest that the multi-nutrient supplement not only helps improve immune function outcomes, but may also provide a beneficial clinical effect in reducing prevalence of infectious disease.

Bogden and colleagues (1994) also conducted a double-blind placebo controlled trial investigating the effect of daily micronutrient supplements on DTH skin responses in healthy older individuals (59-85 years) selected using guidelines of SENIEUR protocol. Twenty-seven subjects were assigned randomly to placebo, and 29 subjects were given an over the counter micronutrient supplement. DTH responses and circulating concentrations of nine micronutrients (ascorbate, retinol, β-carotene, α-tocopherol, copper, zinc, riboflavin, folate and vitamin B_6) were measured before and after 6 and 12 months of supplementation. For the micronutrient group, significant increases were noted in serum concentrations of ascorbate, β-carotene, α-tocopherol, folate and vitamin B_6. At 12 months, in the placebo groups there was an increase in number of subjects with low concentrations for one or more micronutrients. DTH responses to seven recall antigens were significantly increased at 12 months for the micronutrient group but not the placebo

group. The authors suggest that low-to-moderate doses of micronutrients can improve DTH responses in healthy independently living older adults.

In some studies, food or food supplements were used in immunogerontological supplementation. Brosche and Platt (1995) provided 0.5 L of milk daily to 54 older residents from four different homes. Phagocytic activity of peripheral monocytes and granulocytes were measured before and after 4 weeks of milk consumption. Subjects' data were analyzed in 3 groups: A (N=11), B (N=35) and C (N=8), based on subjects with none, one to two, or three pathological values exceeding the limit for three acute-phase parameters, respectively. The acute-phase indicators were leukocyte number, C-reactive protein (CRP), and erythrocyte sedimentation rate (ESR). The results showed an increase in the absolute counts of phagocytosing monocytes in group C, and relative count of phagocytosing monocytes in groups B and C compared to baseline values. Although the mean number of *E. coli* ingested by a single monocyte was unaltered, an increase in the phagocytosis index was seen in group B. The phagocytosis index reflects frequency and phagocytic responsiveness of monocytes. For granulocytes, no significant alterations were noted in phagocytic function. The authors suggest that the immuno-enhancing effect of milk on bacteriophagocytosis of monocytes could be similar to the milk-protein-derived peptides that have protective effects when passed on from mother to host defense of the newborn.

In another study, 47 malnourished subjects (61-97 years) were given appropriate food supplements and followed for six consecutive months (Roebothan and Chandra, 1994). Subjects deficient in iron, folacin or zinc were given daily oral supplement of 50 mg elemental iron, 5 mg folacin or 50 mg elemental zinc, respectively. Vitamin B_{12} (100 µg) was administered monthly in the form of an intramuscular injection. PEM was treated with an oral nutritional supplement drink providing energy, protein, fat, and various vitamins and minerals. Thirty-four subjects completed 6 months of supplementation. DTH skin tests and lymphocyte numbers were assessed before and after supplementation. Mature T cells increased significantly after 6 months

supplementation compared to baseline value, an increase of 13.4% over baseline. However, other immune tests, CD4+ and CD8+ percentages and DTH responses, did not change over 6 months with supplements. Of the 34 subjects, 12 that were diagnosed as zinc-deficient responded more positively to nutritional supplements than any of the other supplemented groups. Changes in immune function in this group were analyzed separately but no statistically significant changes were detected, probably due to the small number of these subjects. Thirty-one subjects completed a subsequent follow up at 12 months. The data for immune function tests at 12 months were compared with those collected after 6 months of supplementation. No significant differences were observed. This small study suggests that poor nutritional status exerts a negative effect on the immune responses of older adults, and at least one aspect of immune function can be improved with proper nutritional status.

1.6 Summary

With the growing proportion of older individuals, maintaining health and well being of this group has become important. This rise in the number of older persons presents unique challenges and opportunities for the scientific community. The prevalence of chronic diseases and infections increase with age, as well as the morbidity associated with these conditions. Such patterns of increased illness in older adults suggest that immune function declines with age. In the past few decades, many immunogerontologists have focused on various aspects of immunity with aging, in order to better understand immune aging. The literature on aging and immunity comes from various observational, experimental and clinical studies, most of which indicate a decline in immunocompetence with age, while some others have not shown such a change.

It is well accepted that nutrition plays an important role in maintaining immune function. Nutrition is also recognized to influence age-related diseases such as osteoporosis, cardiovascular diseases etc. Several studies have been conducted in older adults to investigate nutrition and

immune function outcomes. Studies with single nutrients supplementation have usually been short term, using high doses and small number of subjects. Studies with multiple nutrient supplements have also had some limitations as some have been for short duration or did not include one or more tests of importance such as certain immune tests or data on clinically relevant outcomes such as infection and morbidity. Cross-sectional studies usually determined associations between nutritional variables such as serum micronutrient concentrations and measures of immune function, and no significant associations were found in one large study, the NMAP study. Immune function is influenced by several nutrients and most of the times in older adults multiple nutrient deficiencies coexist at the same time. Very few studies have comprehensively focused on multiple nutrients at the same time and their relationship with immune function (Bogden et al., 1994; Buzina-Suboticanec et al., 1998; Chandra, 1992; Goodwin and Garry, 1988; Goodwin and Garry, 1983; Payette et al., 1990). The studies have focused on correlations between blood levels of nutrients and various immune function tests and/or the effect of multi-nutrient supplements on immune function tests and biochemical indices of nutrient status. To our knowledge, only two of these cross-sectional studies examined the relationship of multiple nutrients statistically at the same time with immune function, using tools such as regression analyses (Goodwin and Garry, 1988; Payette et al., 1990). In one of these studies, Payette et al. (1990) examined the relationship between nutrition and immunologic status by regression analysis in 82 healthy free-living older adults. They included anthropometric and dietary data, blood levels of eight nutrients, hematologic values and plasma fatty acids as the predictive variables; the immune variables included cytotoxicity of natural killer (NK) cells and activity of IL-2. None of the nutrition factors was significantly associated with cytotoxic activity of NK cells ($P<0.05$); however, the dietary intakes of vitamins E and D were negatively associated with the activity of IL-2 ($P<0.007$). The other study was a large study of 230 independently living healthy older men and women of the NMAP study cohort where information on intake and status of several nutrients along with a battery of immune function tests was

obtained for subjects. In this study, Goodwin and Garry (1988) investigated the results of immunologic tests for subjects in the bottom 5%, bottom 10% and top 90% with respect to blood levels of specific nutrients. Their results found no significant associations between malnutrition and depressed immunologic functions in the cohort of 230 independently living healthy older men and women of the NMAP study. However, that study was conducted with an affluent well-educated group where the range of nutrient intakes and status may not have been wide. This study and others do not address the issue of whether certain nutrients together can predict immune function outcomes in the older adults.

Thus, the literature on whether nutrients, examined concurrently in the study cohort, can help to predict immune function with aging is very limited. Because several nutrients can influence immune function and because of interactions among nutrients (Beisel et al., 1981; Fraker et al., 1986; Kaplan and Basford, 1976; Katka, 1984; Kuvibidila and Baliga, 2002; Lesourd, 1995), determining the interactive effects of nutrients on immune function is important. This information could be highly useful from a public health perspective to develop guidelines for maintaining nutritional status and prioritizing nutrient messages to older adults. Therefore, our interest was to examine the relationship between nutritional status of key nutrients involved in maintaining immune response namely protein, iron, zinc, vitamin B_{12}, and folate examined simultaneously with tests of acquired immune function in healthy older adults. Because of blood volume considerations, we focused on protein, iron, zinc, vitamin B_{12}, and folate in the present study as older adults may be at risk for these nutrient deficiencies (Chandra, 2002; Lesourd et al., 1998; Tucker, 1995). Specifically, we wanted to obtain a parsimonious predictive subset of nutrients, which can classify healthy older women, free from inflammation, as low and high responders on several tests of acquired immune response (such as T cells and subsets, lymphocyte proliferation response, and cytokine production) by using the statistical approach of discriminant analysis. Discriminant analysis approach offers the ability to test the final predictive model in

terms of its cross-validation. This implies that discriminant analysis provides estimates of misclassification rates, using the identified predictive model, for classifying subjects into the appropriate groups of high and low responders on the immune function variable. This study is described in Chapter 2.

The associations between the triad of nutrition, immune function and aging are influenced by a variety of factors in the study design such as health status of participants, gender, and several methodological issues concerning assessment. Methodological issues are key as not only is it necessary to consider a panel of comprehensive tests, but also it is important that immune response tests determined yield accurate and precise results. In the last two decades, although significant developments have been made in establishing methodologies for accurate assessment of immune function assays, precision of these tests has not been evaluated thoroughly. Precision relates to the degree of variation in a test, i.e. variability from two main sources, inter- and intra-individual variation. Variation affects assessment and statistical relationships at both the individual and the population level. There is very limited information on variation in immune function tests, particularly on the intra-individual variation. Thus, we also undertook another independent study with the purpose of examining the day-to-day inter- and intra- individual variability in tests of acquired immunity. That study was conducted with generally healthy, well-nourished young and old women, who were selected based on strict subject selection criteria, as poor health and nutritional status are important methodological issues for subject selection that can affect immune response. That study is described in Chapter 3.

1.7 References

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CHAPTER 2

NUTRITION, T CELL FUNCTION AND CYTOKINE PRODUCTION IN HEALTHY OLDER WOMEN

2.1 Introduction

Aging is often associated an increased susceptibility to infections and cancer, in addition to the morbidity and mortality associated with these conditions (Makinodan, 1995). Although a number of factors contribute to these conditions, the age-related changes in various components of the immune system have been thought to play a very important contributory role. Many studies have shown that *in vivo* tests such as DTH responses, or *in vitro* tests such as proliferation of peripheral blood mononuclear cells with mitogens or activity of NK cells and phagocytes are associated with or can predict morbidity and mortality (Ginaldi et al., 1999; Murasko et al., 1987; Niwa et al., 1989; Wayne et al., 1990).

Evidence from many clinical and experimental studies in humans and animals suggest a decline in immune function with aging. An important limitation in many of these studies is the absence of strict subject selection criteria such as the SENIEUR protocol (Ligthart et al., 1984). The SENIEUR protocol is a standardized set of criteria for subject selection in immunogerontological studies based on clinical and laboratory data, which allows for selection of 'healthy' subjects without any underlying disease or inflammatory conditions. Thus, some of the age-associated declines that have been reported without the use of SENIEUR protocol (Hallgren et al., 1988; Murasko et al., 1987; Polignano et al., 1994; Rabatic et al., 1988; Schwab et al., 1990; Wikby et al., 1994) can be the result of disease, inflammation or infection, and not necessarily because of the aging process itself. Many of the recent immunogerontological studies have acknowledged the use of strict selection criteria in their study design. However, even with the use of SENIEUR protocol, age-associated declines in immune function are noted (Cakman et al., 1996; Facchini et al., 1987; Nijhuis et al., 1994; Ogata et al., 1997; Sansoni et al., 1993; Vitale et al., 1992). Therefore, it may be possible that other factors such as nutritional status, particularly nutritional deficiencies will contribute to the decline in immune function tests. It is

important to note that the only nutrition criterion included in the SENIEUR protocol body mass index (BMI).

Nutrition is important for immunocompetence and can affect the risk of various diseases (Bell and High, 1997; Bhaskaram, 2002; Chandra, 2002). Adequate status of both macronutrients (energy and protein) and micronutrients (Vitamins A, E, B_6, B_{12}, folate and C, iron, and zinc) is important in mounting optimal immune response (Beisel et al., 1981; Chandra, 1991). There is vast literature suggesting that specific nutrient deficiencies can also affect immune function (Beisel et al., 1981, Katka, 1984; Kuvibidila and Baliga, 2002; Lesourd, 1995; Meydani and Beharka, 1996; Shankar and Prasad, 1998). Nutrient deficiencies can, therefore, contribute further to the age-related decline in immune function in older individuals, and can increase the risk of infections, illnesses and associated morbidities (Chandra, 1992; Lesourd, 1995; Vellas et al., 1997). Thus maintaining optimal nutritional status with aging may offer a means to maintaining immune function (Krause et al., 1999; Lesourd et al., 1998).

Several studies have investigated the relationship between nutrition and immune function in older adults. Most employed a study design whereby a single nutrient deficiency such as vitamin B_6 (Meydani et al., 1991), vitamin B_{12} (Fata et al., 1996), protein (Lesourd, 1995), zinc (Allen et al., 1983), or iron (Ahluwalia et al., 2003) was examined. Some researchers have also examined the effects of supplementation with single nutrients on immune function in older adults (Bogden et al., 1990; Corridan et al., 2001; Meydani et al., 1990). The results of such studies indicate that nutrient deficiencies are associated with impairments in some aspects of immune function and the correction of nutrient deficiency is usually associated with improvement in immune response. Another methodology often employed in examining the relationship of nutrition and immune function is supplementation trials with multiple micronutrients. These studies in older adults have demonstrated either beneficial (Buzina-Suboticanec et al., 1998;

Chandra, 1992) or adverse (Bogden et al., 1990; Fortes et al., 1998) or no effects (Goodwin and Garry, 1983; Murphy et al., 1992).

Immune function is influenced by several nutrients and most of the times in older adults, multiple nutrient deficiencies coexist at the same time (Chandra, 2002; Lesourd et al., 1998; Tucker, 1995). It is interesting to note, however, that most studies examining the relationship of nutrition and immune function in older adults have focused on either single nutrients (Lesourd, 1995; Meydani et al., 1990; Meydani et al., 1991) or with multiple nutrients, yet utilized univariate analyses to examine the relationship of one nutrient at a time with immune functions of interest (Buzina-Suboticanec et al., 1998; Chandra, 1992; Fülöp et al., 1999). Thus, few studies have examined the relationship of body's status of several nutrients examined simultaneously with immune function in older adults (Bogden et al., 1994; Buzina-Suboticanec et al., 1998; Chandra, 1992; Goodwin and Garry, 1988; Goodwin and Garry, 1983; Murasko et al., 1987; Payette et al., 1990).

Most of these investigators examined immune function tests in subjects with varied nutritional status, before and after multiple nutrient supplementation, using statistical tools such as ANOVA or t tests; or they compared subjects with adequate versus those with inadequate status for multiple nutrients in a cross-sectional manner using statistical tools such as correlations, t tests or chi-square. To our knowledge, only two cross-sectional studies concerning the relationship of several nutrients simultaneously with immune function, have used tools such as regression analyses (Goodwin and Garry, 1988; Payette et al., 1990). In one of these studies, Payette et al. (1990) examined the relationship between nutrition and immunologic status by regression analysis in 82 healthy free-living older adults. They included anthropometric and dietary data, blood levels of eight nutrients, hematologic values and plasma fatty acids as the predictive variables; the immune variables included cytotoxicity of natural killer (NK) cells and activity of IL-2. None of the nutrition factors was associated with cytotoxic activity of NK cells;

however, the dietary intakes of vitamins E and D were negatively associated with the activity of IL-2. The other study was a large study of 230 independently living healthy older men and women of the New Mexico Aging Process (NMAP) study cohort where information on intake and status of several nutrients along with a battery of immune function tests was obtained for subjects. In this study, Goodwin and Garry (1988) took a unique approach by comparing the results of immunologic tests for those subjects in the bottom 5%, bottom 10% and top 90% with respect to blood levels of specific nutrients. No significant associations between malnutrition and depressed immunologic functions. However, this study was conducted with an affluent, well-educated group of older persons where the range of nutrient intakes and status may not have varied. Furthermore, this study and others do not address the issue of whether certain nutrients examined together can predict immune function outcomes in older adults.

Thus, literature on whether nutrients, examined concurrently in the study cohort, can help to predict immune function with aging is very limited. It is of interest to examine the relationship of several nutrients involved in maintaining immune response with immune function in older adults for several reasons: nutrient deficiencies usually coexist in older persons (Chandra, 2002; Lesourd et al., 1998; Tucker, 1995); several nutrients can influence immune function; and nutrient interactions can be important (Beisel et al., 1981; Fraker et al., 1986; Kaplan and Basford, 1976; Katka, 1984; Kuvibidila and Baliga, 2002; Lesourd, 1995). Therefore, we were interested in examining the relationship between statuses of key nutrients known to be involved in maintaining immune response examined simultaneously with various aspects of acquired immunity. Because of blood volume considerations, we focused on protein, iron, zinc, vitamin B_{12}, and folate in the present study as older adults may be at risk for these nutrient deficiencies (Chandra, 2002; Lesourd et al., 1998; Tucker, 1995). Our interest was to identify which, if any, of key nutrients known to affect immune function namely protein, iron, zinc, vitamin B_{12}, and folate, could predict immune function in a cohort of healthy older women free from

inflammation. We used the statistical approach of discriminant analysis to identify a parsimonious predictive subset of laboratory tests of nutrient status, which can correctly classify healthy older women as low and high responders on several tests of acquired immune response such as T cells and subsets, lymphocyte proliferation response, and cytokine production. The discriminant analysis approach was used because not only does it enable us to develop an optimal predictive model but also it offers the ability to test the predictive model in terms of its cross validation. In other words, using the approach of discriminant function analysis, estimates of misclassification rates for classifying subjects using the identified predictive model (subset of nutrients) into their appropriate groups (high or low responder on the immune function variable) can be estimated. This approach allows investigators to examine how well the identified discriminant function of the predictive model works (keeping in mind that a perfect predictive model identified by discriminant analysis would provide a misclassification rate of 0%). This information could be highly useful from a public health perspective to develop guidelines for maintaining nutritional status and prioritizing nutrient messages to older individuals.

2.2 Methods

2.2.1 Subject recruitment

Older women (> 60 y) were recruited with the assistance of the Agencies on Aging from Center, Blair, and Clearfield counties; and from local housing complexes for seniors using flyers, advertisements, and recruitment letters describing the study. Women between the ages of 41 and 59 years were not included in the study because of the potential confounding effects of hormonal changes associated with menopause on immune parameters (Hough et al., 1999; Ligthart et al., 1984; Murasko et al., 1987; Porter et al., 2001). Women receiving hormone replacement therapy were also not included in the study (Porter et al., 2001). Subjects provided written informed consent for participation and followed protocols approved by the Office for Regulatory

Compliance at the Pennsylvania State University. Subjects received an honorarium of $100 at the completion of the study. The flowchart for the overall subject recruitment and the screening protocol is presented in Figure 2-1.

2.2.2 Study protocol

The data presented in this paper are the pooled data of women from two separate studies conducted in our laboratory. Study 1, conducted from 1997-99, focused on nutrition and immune response in young and old healthy well-nourished women (n=71). Study 2 data was collected from 1999 to 2002 on 66 older women; the focus of this study was examining the relationship of nutrition status and immune response in older women. The methodologies used in both studies were similar except for the fact that in the latter study, 2 days data on immune function variables were collected for each subject. The flowchart for the overall study protocol is presented in Figure 2-2.

Approximately 180 older women were screened for participation in the study. A verbal medical history was taken to rule out any health conditions or medication use as per the SENIEUR protocol (Ligthart et al., 1984). Of the 156 women who met the medical history screen, 137 provided fasting blood samples between the hours of 7 a.m. and 9 a.m., following a 15-minute period of rest. A certified phlebotomist collected approximately 35 mL of venous blood into 3 types of Vacutainer® tubes (Becton Dickinson, Franklin Lakes, NJ): tube with no anticoagulant, tube containing EDTA, and tube containing heparin. Height and weight were also recorded using portable, standardized instruments.

Next, women were screened for inflammation based on laboratory tests, erythrocyte sedimentation rate (ESR), serum alpha-1 acid glycoprotein (AGP), and white blood cell (WBC) count. Subjects presenting elevated levels for any of the 2 of 3 inflammatory tests were considered to have inflammation and were dropped from further participation (n=7, as described

below). Therefore, the final number of subjects who were eligible to continue participation in the study was 130 (IDs for the final study cohort are presented in Appendix D). Immune function tests were carried out for these subjects on day 1 blood collection. An additional blood sample (25 mL) was obtained whenever possible from subjects (n=61) within a week of the first blood draw, to determine tests of immune function and obtain a complete blood count (CBC).

2.2.3 Screening Criteria

Medical history and clinical tests of general health and inflammatory status were used to exclude individuals with acute or chronic inflammation, other medical conditions, and/or medication use known to affect immune response, in accordance with the SENIEUR protocol (Ligthart et al., 1984). A CBC with differential evaluation on a Coulter MAZM analyzer (Beckman Coulter Corporation, Miami, FL) and clinical chemistry tests (Chem-24 profile) using a Roche Mira Plus random access chemistry analyzer (Roche Boehringer Mannheim Corporation, Indianapolis, IN) were carried out at the University Health Services Laboratory. Results of these tests were reviewed by the study physician (Dr. Gordon Handte) to exclude subjects with infection, inflammation, liver disorders, kidney disorders, and/or bone marrow proliferation disorders.

To assess inflammatory status, ESR was measured using the Westergren Method (Dispette72, Ulster Medical Products, Rio Rancho, NM). Serum AGP was determined by radial immunodiffusion (Kent Laboratories, Redmond, WA) and controls provided by Kent Laboratories were used for each plate assayed. Further, elevated WBC count from the CBC was also considered for evaluation of inflammation. The cut-offs for defining abnormal results for these parameters were: ESR > 30 mm/h for old individuals; WBC > 11 x 10^9/L; and AGP > 1.4 g/L (Jacobs et al., 2001). Women with abnormal results on two or more of these tests of inflammation were excluded from further participation.

2.2.4 Nutritional Status

To assess nutritional status, laboratory tests were performed to determine protein, iron, zinc, vitamin B_{12}, and folic acid status. Serum total protein and serum albumin were determined as part of the clinical chemistry profile. Serum ferritin was assayed by a radioimmunoassay (Diagnostic Products Corporation, Los Angeles). Serum transferrin receptor (TfR) was assayed using a commercial ELISA (Ramco Laboratories, Houston, TX). Hemoglobin, hematocrit, mean corpuscular volume (MCV), and red cell distribution width (RDW) were obtained from the CBC analysis. Serum vitamin B_{12} and folate concentrations were determined by a commercial radioimmunoassay (ICN Pharmaceuticals, Orangeburg, NY). Pooled serum from ongoing studies in our laboratory were used as internal controls for each batch of samples that were analyzed for serum ferritin, serum transferrin receptor, serum vitamin B_{12} and folate. Plasma zinc was measured by atomic absorption spectrophotometry.

Subjects with BMI < 20, serum total protein < 60 g/L and/or serum albumin < 35 g/L (n=28) were considered to have low protein status (Jacobs et al., 2001). Individuals with depleted iron stores (n=5); based on serum ferritin < 20 μg/L for old women (Ahluwalia et al., 1995; Milman et al., 1983); or tissue iron deficiency (serum TfR > 8.5 mg/L) (n=4); or iron deficient erythropoiesis (serum ferritin < 20 μg/L or serum TfR > 8.5 mg/L, and at least 1 abnormal test of iron transport (serum iron < 500 μg/L, TIBC > 3500 μg/L, and TS < 16 %) (n=12); or iron deficiency anemia (iron deficient erythropoiesis and evidence of red cell changes indicating anemia with hemoglobin < 120 g/L, or hematocrit < 36 % or mean cell volume < 80 fL) (n=12), were considered to have low iron status. Subjects who had plasma zinc levels < 700 μg/L (n=7) were considered to have low zinc status (Gibson, 1990). Subjects with serum vitamin B_{12} < 250 ng/L, serum folate < 2 μg/L (Carmel, 1988; Jacobs et al., 2001) and/or mean corpuscular volume > 102 fL (n=12) (Hillman and Finch, 1996) were considered to have low vitamin B_{12} and folate

status. IDs who were considered to have low status, based on the cutoffs mentioned above, for protein, iron, zinc, vitamin B_{12} and folate are presented in Appendices E-H.

2.2.5 Immune function tests

For sample volume considerations, immune function tests were determined using whole blood assays previously validated in our laboratory (Krause et al., 1999).

2.2.5.1 T cells and subsets

T cells and subsets were estimated in 1 mL heparinized blood (Mandy et al., 1997) using fluorescently-labeled, monoclonal antibodies specific for surface antigens (anti-CD3 recognizes total T-cells; anti-CD4 recognizes T-helper cells, and anti-CD8 recognizes T-cytotoxic cells) by flow cytometry (Beckman Coulter EPICS XL, FL). Total lymphocyte number, obtained from the CBC with differential evaluation, was used to compute the absolute numbers of cells and for obtaining monocytes and granulocytes number.

2.2.5.2 T-cell proliferation response to stimulation with mitogens

Proliferation of T-cells in response to two concentrations each of phytohemagglutinin (PHA) and concanavalin A (Con A) were determined by measuring incorporation of [^{3}H] thymidine (6.7 Ci/mmol) (Bloemena et al., 1989; Fletcher et al., 1987). The two optimal concentrations assayed for PHA were 5 and 10 mg/L and those for Con A were 12 and 25 mg/L, respectively, based on our previous study (Krause et al., 1999). Heparinized blood was diluted (1:10) with RPMI 1640 medium containing 10% fetal bovine serum (FBS), L-glutamine (2 mmol/L), penicillin (100,000 U/L), and streptomycin (100 mg/L). One hundred µL diluted blood and 100 µL of mitogens were added to wells in a 96-well, round-bottom microtitre plate. For each mitogen at each concentration, six replicate measurements were carried out per subject on

each day of blood collection. Cells were incubated for 42 h (humidified, 5% CO_2, 37 °C) followed by addition of 10 μL (1 μCi) of [^{3}H]thymidine to each well, and further incubation for another 6 h, followed by harvesting of cells onto glass fiber filters (Skatron 7025 Combi Cell Harvester, Skatron Inc., Sterling, VA). Incorporation of [^{3}H]thymidine (CPM) into cellular DNA was determined with a Wallac 1205 beta plate counter (EG&G Wallac Inc., Gaithersburg, MD). CPM was normalized to 1000 T cells.

2.2.5.3 Cytokine determination

Heparinized blood was diluted (1:10) with RPMI 1640 medium containing 10% FBS, L-glutamine (2 mmol/L), penicillin (100,000 U/L), and streptomycin (100 mg/L). For each subject, 2 mL of diluted blood with or without PHA (5 mg/L; PHA-M {L2646} obtained from Sigma, St. Louis, MO) was placed in the wells of a 24-well, flat-bottom microtitre plate. Cells were incubated for 48 h (humidified, 5% CO_2, 37 °C). Contents of wells were transferred into microfuge tubes, centrifuged, and supernatants collected, aliquotted, and frozen at -80 °C until subsequent assay of IL-1β, IL-2 and IL-6 as described previously (Ahluwalia et al., 2001).

Cytokines were assayed with an ELISA. Samples from day 1 for each subject were assayed on the same plate and a pooled supernatant was used as the internal control with each run. The pooled supernatant was generated by culturing the blood of several individuals separately with PHA (as described above) and pooling the supernatants. Three replicate measurements were determined for each cytokine measurement on each day of blood collection per subject. Cytokines (IL-1β, IL-2, and IL-6) were measured by ELISA, using antibodies obtained from R&D systems. Briefly, supernatants from cell cultures with PHA were added to anti-human monoclonal IL-2 antibody coated plates (MAB602) and detected with a polyclonal goat anti-human IL-2 antibody (BAF 202). The absolute amount of IL-2 from total T (IL-2 pg/1000 T cells) and T helper cells (IL-2 pg/1000 T helper cells) was computed from pg/ml of the

culture supernatant. Similar to IL-2, IL-1β concentration was determined in supernatants using anti-human monoclonal IL-1β antibody coated plates (MAB601) and detected with a polyclonal goat anti-human IL-1β antibody (BAF201). IL-1β (pg) per 1000 granulocytes and IL-1β (pg) per 1000 granulocytes plus monocytes were computed from IL-1β concentration determined from culture supernatant (pg/mL). For IL-6 determination, culture supernatants were added to anti-human monoclonal IL-6 antibody coated plates (R&D systems # MAB206) and detected with a polyclonal goat anti-human IL-6 antibody (R&D systems # BAF206). IL-6 (pg) per 1000 granulocytes and IL-6 (pg) per 1000 granulocytes plus monocytes were computed from IL-6 concentration determined from culture supernatant (pg/mL). The lower limit of detection for all cytokines measured was 3 pg/mL. Cytokine levels in the supernatants were usually nondetectable in the absence of PHA.

2.2.6 Statistical analyses

Statistical analyses were carried out using the Statistical Analysis System version 8.0 (SAS Institute, Cary, NC). Most of the nutrition variables were consistent with a normal distribution, except serum ferritin. Therefore, logarithmic transformed data were used for serum ferritin. For immune function variables, only absolute numbers of T cells and subsets were consistent with normality, therefore, logarithmic transformed data were used for all lymphocyte proliferation response and cytokine variables. In study 2, immune function data was obtained on two days. Paired t-test for these variables between day 1 and day 2 data were not significant (Appendix I), therefore data were collapsed over the two days and average of data from day 1 and day 2 for immune function tests was used for study 2 and merged with one day data from study 1 to arrive at the final data set for statistical analysis.

The immune function variables examined in this study were: T cells and subsets (Total T cells, T helper cells, T cytotoxic cells), lymphocyte proliferation response (LPR) to PHA (5 and

10 mg/L) and Con A (12 and 25 mg/L), and cytokine production [IL-2 pg / 1000 T cells, IL-2 pg / 1000 T helper cells; IL-1 pg / 1000 granulocytes, IL-1 pg / 1000 granulocytes and monocytes; and IL-6 pg / 1000 granulocytes, IL-6 pg / 1000 granulocytes and monocytes]. For each immune function variable (T cell and subsets, LPR, and cytokines) first subjects were classified as low and high responders on that immune function test. Subjects in the low responders groups had values for the immune variable ≤ 25th percentile of the study cohort, whereas, subjects in the high responders group for the immune variable ≥ 75th percentile of the study cohort. Discriminant analysis approach was used to identify an optimal predictive subset of nutritional status variables that could classify subjects into low and high responders for each immune function test with a high degree of precision (low misclassification rate). The nutrition status variables included BMI, serum protein and albumin for protein status; serum ferritin, serum TfR, transferrin saturation, hemoglobin, hematocrit, MCV, RDW for iron status; plasma zinc, and serum levels of vitamin B_{12} and folic acid for status of zinc, vitamin B_{12} and folic acid, respectively.

Both PROC DISCRIM and PROC STEPWISE procedures were used to carry out the discriminant analysis. Preliminary tests done using PROC UNIVARIATE and PROC DISCRIM indicated that the assumptions of normality and equal covariance matrixes were met to warrant the use of the linear discriminant function. For prediction purposes, a parsimonious set of variables were chosen from the nutrition variables by using the following four criteria as per the approach used in a previous study (Ahluwalia et al., 1995):

- Evidence suggested by the literature
- A large and significant “F to enter”, which relates to the significance of the ordinary F statistic for group differences in a 1-way analysis of variance (ANOVA). A large “F to enter” indicated that the means of the two groups were far apart relative to the (pooled) within-group variation. The significance level of 0.20 was used based on the results of Costanza and Affifi (Costanza and Affifi, 1979).

- A larger and significant "F to remove", relating to the separation of the means of the two groups after adjustment for the presence of the other dependant variables in the model (Ahluwalia et al., 1995).
- The order of entry of the variables in a forward stepwise discriminant analysis, whereby variables that are not significant, i.e. P>0.20, were excluded from consideration (Costanza and Affifi, 1979).

Variables ranked highly using these four criteria were selected as the near model of predictive subset. There is no true optimal subset in a statistical sense, as different subsets may provide very similar prediction, where prediction is used in the sense of using the discriminant analysis equations to correctly classify individuals into one of the two groups. The optimal subset of variables (final model) was selected from this near model based on crossvalidation results (using PROC DISCRIM option CROSSVAL and POSTERR in SAS) because this parsimonious subset is likely to give a better predictive subset in a cross-validation sense, i.e. the performance of the linear discriminant function was evaluated using the 'leave-out-one' procedure of Lachenbruch and Morrison (Lachenbruch, 1975; Morrison, 1990). This procedure classifies each subject by using a discriminant function computed from the other data points in the data set, excluding the observation being classified. The CROSSVAL and POSTERR options in SAS DISCRIM were used to carry out the 'leave-out-one' cross-validation procedure and obtain error rates for misclassification.

In summary, discriminant analysis procedure provided an optimal predictive subset from various nutrition variables to classify groups as high and low responders for immune function variables. The linear discriminant functions for classifying individuals into the two groups were computed and evaluated for their performance in classifying subjects into the high and low groups and estimates for misclassification rates were computed.

In addition as secondary analysis, Pearson's correlation was used to determine relations between the nutrition and immune function variables. Further, ANOVA was used to compare subjects with varying BMI status (normal, overweight and obese) on all the immune function variables.

2.3 Results

The description of the study cohort is presented in Table 2-1. Most of the women were homebound, non-obese and had varied nutritional status (Table 2-1). For all immune function variables, the predictive subset of variables was selected in the model using the four criteria of discriminant analysis described in section 2.2.6. Results of discriminant analysis are presented in three sections for each set of immune function variables: T cells and subsets, lymphocyte proliferation response to mitogens, and cytokine production: For each of these sets of immune function variables we first present the differences in the means of low and high immune function groups on the nutrition variables indicated by the F statistic ('F to enter') for group differences obtained using ANOVA ($P<0.2$). This served as one of the 4 criteria for building the optimal predictive subset of nutrients for classifying individuals into their group (high or low) on various immune function tests. The final predictive discriminant model is presented for each of these immune function variables.

2.3.1 T cell and subsets

Tables 2-2, 2-3 and 2-4 present the differences between high and low responders on number of total T (CD3+), T helper (CD4+) and T cytotoxic (CD8+) cells, respectively. The low and high responder groups on T cells and subsets did not differ significantly on any of the nutrition variables examined in a univariate manner (Tables 2-2, 2-3 and 2-4; ANOVA; $P<0.05$). Table 2-5 presents the predictive discriminant models for total T and T subsets. Interestingly,

protein, iron, and zinc were identified as predictive model for all three T cells. The misclassification rates based on this predictive subset of nutrients was highest for CD3+ numbers (37.2%) and lowest for CD4+ numbers (16.5%) (Table 2-5). Thus, the probability of correctly classifying subjects into high or low CD3+, CD4+ and CD8+ groups by using the discriminant subset of protein, iron and zinc status variables ranged from 62.8-83.5%.

2.3.2 Lymphocyte proliferation response to PHA and Con A

The differences between high and low responders on lymphocyte proliferation response (cpm / 1000 T cells) to PHA at concentrations of 5 and 10 mg/L and Con A at concentrations of 12 and 25 mg/L are provided in Tables 2-6, 2-7, 2-8, and 2-9, respectively. For, lymphocyte proliferation variables the low and high responder groups for PHA 10 and Con A 12 differed on MCV (Tables 2-7 and 2-8; $P<0.05$); whereas Con A 25 groups differed on BMI and zinc (Table 2-9; $P<0.05$).

Table 2-10 presents the predictive discriminant models for these lymphocyte proliferation related variables. For lymphocyte proliferation response to PHA at concentrations of 5 and 10 mg/L, protein and iron were identified in the final predictive models, and provided misclassification rates of 15.4% and 20.7 %, respectively (Table 2-10). For lymphocyte proliferation response to Con A at concentrations of 12 and 25 mg/L, protein, iron, and zinc were identified in the final predictive models, and provided misclassification rates of 10.3% and 15 % (Table 2-10), respectively, which were lower than that for PHA. Therefore, the probability of correctly classifying each subject into high or low responder on lymphocyte proliferation response to PHA, using the predictive subsets based on protein and iron status variables was fairly high and ranged between 79.3-84.6%. Similarly, the probability of correctly classifying each subject into high or low responder on lymphocyte proliferation response to Con A, using the

predictive subsets based on protein, iron and zinc status variables was high and ranged between 85-89.7%.

2.3.3 Cytokines

The differences between high and low responders on T cell cytokine IL-2 and proinflammatory cytokines, IL-1 and IL-6 are presented in Tables 2-11 through 2-16. As for T cells and subsets and the lymphocyte proliferation response to mitogens, the low and high groups for cytokine production no consistent differences were seen with respect to nutrition variables examined (Tables 2-11 through 2-16; $P<0.05$). However, the IL-2 high and low groups differed significantly on hemoglobin and vitamin B_{12} (Tables 2-11 and 2-12; $P<0.05$); the IL-1 high and low groups differed on vitamin B_{12} (Tables 2-13 and 2-14; $P<0.05$); and the IL-6 high and low groups differed significantly on serum albumin and transferrin saturation (Tables 2-15 and 2-16; $P<0.05$).

The final discriminant predictive model and associated error rates for classification into groups for all three cytokines are presented in Table 2-17. Interestingly, for IL-2 groups, protein, iron, and vitamin B_{12} were identified as predictive variables in the final model and provided lowest misclassification rates, 13.4% and 11.1%, compared to the other models (Table 2-17). For IL-1 and IL-6 groups, protein, iron, vitamin B_{12} and folate were identified in the final predictive models, and provided misclassification rates ranging from 17.5-22.2% (Table 2-17). Therefore, the probability of correctly classifying each subject into high or low responder on IL-2 variables, using the predictive subsets based on protein, iron and vitamin B_{12} status variables was highest compared to other cytokines and ranged from 86.6-88.9%. For IL-1 and IL-6, the probability of correctly classifying each subject into high or low responder on the cytokine variable, using the predictive subsets based on protein, iron, vitamin B_{12}, and folate status variables was also high, 77.8-82.5%.

2.3.4 Summary of results

A summary of all the final discriminant models for each immune function variable is provided in Table 2-18. It is interesting to note that protein and iron status variables were identified in the predictive subset for all immune function variables examined; zinc emerged in the final predictive subset for several acquired immune function variables, namely T cells and subsets, and lymphocyte proliferation response to Con A. However, vitamin B_{12} and folate were identified in the final predictive subset only for cytokine variables. As part of secondary analysis, the relationships between the nutrition and immune function variables were determined by correlation (Table 2-19). Most of the nutrition and immune function variables were not correlated on a single nutrient basis. However, few associations were noted. Serum albumin, serum transferrin receptor, hemoglobin, plasma zinc and serum vitamin B_{12} were significantly correlated with IL-2 variables; and serum albumin, transferrin saturation, mean cell volume and red cell distribution width were significantly correlated with IL-6 variables. In addition, two of the lymphocyte proliferation response variables (PHA, 5 mg/L and Con A, 12 mg/L) were significantly correlated with some of the iron status variables.

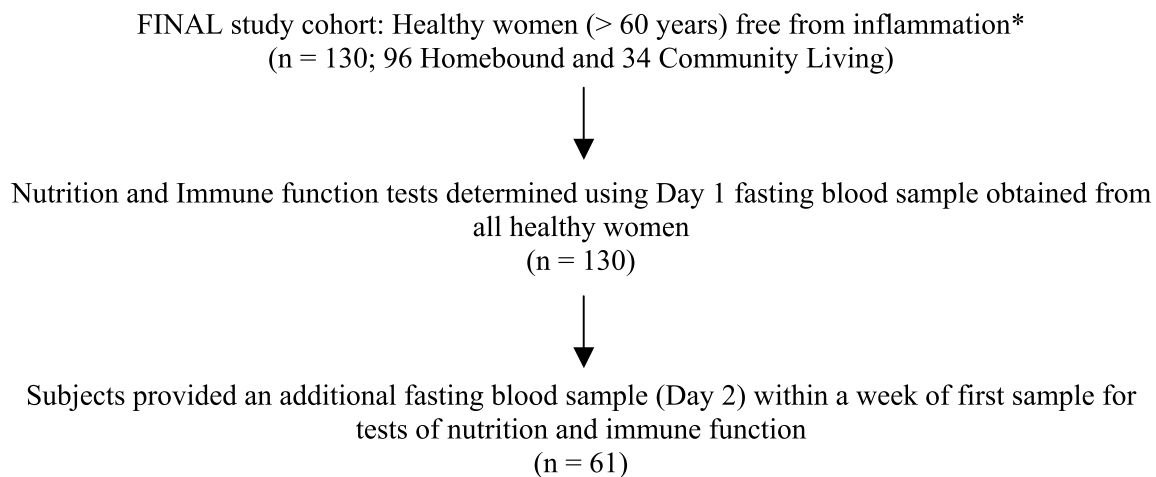
Figure 2-1. Overall flowchart for subject recruitment and screening protocol

*Screening was carried out using clinical tests:

- CBC and clinical chemistries to rule out systemic disease
- Inflammation was considered present if 2 of 3 tests were positive: ESR > 30 mm/h; WBC > 11 x 10^9/L; and AGP > 1.4 g/L

**Data on 43 women were collected by the author and Amy Miltko

Figure 2-2. Overall flowchart for study protocol



*Total N is the pooled data from study 1 (n = 69) and study 2 (n = 61)
Subjects recruited by the author and Amy Miltko: (n = 43 in study 2)

Table 2-1. Description of study participants

Variable	Number of subjects (%)
Place of Residence	
Community living	34 (26.1)
Homebound	96 (73.9)
Protein Status	
Inadequate	28 (21.5)[a]
Adequate	102 (78.5)
Iron Status	
Inadequate	33 (25.4)
Adequate	97 (74.6)
Zinc Status	
Inadequate	7 (5.4)
Adequate	123 (94.6)
Vitamin B_{12} and folate Status	
Inadequate	12 (9.2)
Adequate	118 (90.8)
Age	76.66 ± 6.95[b]

[a]Criteria and cutoffs used to define nutrient status are provided in section 2.2.4.
[b]Mean ± SD

Table 2-2. Descriptive and nutrition variables by total T cells (CD3+) group

Variables	**Low CD3+ group**[a] **(Cut-off: < = 1.0178) (n = 30)**	**High CD3+ group (Cut-off: > = 1.711) (n = 31)**	**P value**
CD3 number (x 10^9/L)	0.73 ± 0.16[b]	2.33 ± 0.66	**< 0.0001**
Age	78.53 ± 6.17	75.68 ± 7.4	0.1075
Nutrition variables			
Body mass index (kg/m^2)	25.38 ± 5.51	25.98 ± 5.43	NS
Protein (g/L)	68.0 ± 6.0	69.4 ± 6.0	NS
Albumin (g/L)	40.6 ± 3.3	41.6 ± 3.7	NS
Serum ferritin (µg/L)	47.86 (16.98 - 134.9)[c]	75.86 (33.88 - 169.82)	NS
Serum transferrin receptor (mg/L)	6.69 ± 2.34	6.23 ± 2.51	NS
Transferrin saturation (%)	25.17 ± 10.48	26.13 ± 9.11	NS
Hemoglobin (g/L)	132.2 ± 12.2	135.4 ± 15.4	NS
Hematocrit (%)	39.56 ± 3.38	40.78 ± 4.78	0.0986
Mean cell volume (fL)	92.60 ± 5.1	91.29 ± 5.81	NS
Red cell distribution width (units)	13.29 ± 1.22	13.20 ± 1.06	0.0697
Plasma zinc (µg/L)	882.7 ± 168.6	908.1 ± 150.2	NS
Serum vitamin B_{12} (ng/L)	568.39 ± 244.91	560.02 ± 286.15	NS
Serum folate (µg/L)	20.87 ± 11.83	18.52 ± 8.65	NS

[a]Groups are defined based on percentiles (low ≤ 25th and high ≥ 75th) of absolute CD3+ number
[b]Values are mean ± SD
[c]Values are geometric mean and values in parentheses indicate ± 1 SD of geometric mean

Table 2-3. Descriptive and nutrition variables by T helper cells (CD4+) group

Variables	**Low CD4+ group**[a] **(Cut-off: < = 0.6012) (n = 31)**	**High CD4+ group (Cut-off: > = 1.14003) (n = 31)**	**P value**
CD4 number (x 10^9/L)	0.46 ± 0.14[b]	1.54 ± 0.38	**< 0.0001**
Age	78.68 ± 5.68	75.71 ± 7.3	0.0791
Nutrition variables			
Body mass index (kg/m^2)	24.57 ± 5.76	26.93 ± 5.64	0.1068
Protein (g/L)	67.7 ± 4.4	69.3 ± 5.8	NS
Albumin (g/L)	41.0 ± 3.5	41.4 ± 3.5	NS
Serum ferritin (µg/L)	58.88 (24.54 - 141.25)[c]	66.07 (25.19 - 173.78)	NS
Serum transferrin receptor (mg/L)	6.50 ± 2.25	6.29 ± 2.33	NS
Transferrin saturation (%)	24.74 ± 9.05	24.94 ± 8.53	NS
Hemoglobin (g/L)	134.9 ± 11.7	135.8 ± 15.5	NS
Hematocrit (%)	40.18 ± 3.12	40.84 ± 4.83	NS
Mean cell volume (fL)	92.79 ± 4.85	91.47 ± 5.6	NS
Red cell distribution width (units)	13.15 ± 1.16	13.11 ± 0.89	NS
Plasma zinc (µg/L)	935.5 ± 197.1	890.7 ± 138.3	NS
Serum vitamin B_{12} (ng/L)	597.58 ± 255.73	539.07 ± 263.42	NS
Serum folate (µg/L)	21.16 ± 9.48	19.19 ± 8.43	NS

[a]Groups are defined based on percentiles (low ≤ 25th and high ≥ 75th) of absolute CD4+ number
[b]Values are mean ± SD
[c]Values are geometric mean and values in parentheses indicate ± 1 SD of geometric mean

Table 2-4. Descriptive and nutrition variables by T cytotoxic cells (CD8+) group

Variables	**Low CD8+ group[a] (Cut-off: < = 0.26671) (n = 31)**	**High CD8+ group (Cut-off: > = 0.63215) (n = 31)**	**P value**
CD8 number (x 10^9/L)	0.18 ± 0.07[b]	0.91 ± 0.37	**< 0.0001**
Age	77.35 ± 7.67	76.87 ± 7.57	NS
Nutrition variables			
Body mass index (kg/m^2)	25.43 ± 5.33	25.5 ± 6.87	NS
Protein (g/L)	69.7 ± 5.4	68.9 ± 5.9	NS
Albumin (g/L)	42.3 ± 2.8	41.7 ± 3.9	NS
Serum ferritin (μg/L)	51.28 (16.98 - 154.88)[c]	50.18 (14.79 - 169.82)	NS
Serum transferrin receptor (mg/L)	6.5 ± 2.48	5.96 ± 2.11	NS
Transferrin saturation (%)	24.97 ± 10.59	25.81 ± 10.23	NS
Hemoglobin (g/L)	131.1 ± 11.5	136.6 ± 15.2	0.1126
Hematocrit (%)	39.44 ± 3.46	40.98 ± 4.72	0.1492
Mean cell volume (fL)	91.55 ± 5.37	90.6 ± 6.33	NS
Red cell distribution width (units)	13.28 ± 1.28	13.23 ± 0.96	NS
Plasma zinc (μg/L)	999.8 ± 245.4	929.4 ± 186.0	NS
Serum vitamin B_{12} (ng/L)	572.74 ± 283.65	507.03 ± 273.29	NS
Serum folate (μg/L)	17.62 ± 6.77	17.88 ± 8.66	NS

[a]Groups are defined based on percentiles (low ≤ 25th and high ≥ 75th) of absolute CD8+ number
[b]Values are mean ± SD
[c]Values are geometric mean and values in parentheses indicate ± 1 SD of geometric mean

Table 2-5. Discriminant Analysis Final Model for Classification of Individuals into Low and High Responders for T cells and Subsets

Final models	Variables selected in the model	Misclassification rate (%)
CD3+ number (x 10^9/L)	Protein Serum ferritin Mean corpuscular volume Plasma zinc	37.2
CD4+ number (x 10^9/L)	Body mass index Protein Hemoglobin Hematocrit Plasma zinc	16.5
CD8+ number (x 10^9/L)	Albumin Hemoglobin Mean corpuscular volume Plasma zinc	25.4

Table 2-6. Descriptive and nutrition variables by phytohemagglutinin A (5 mg/L) group

Variables	Low Phytohemagglutinin A (5 mg/L) group[a] (Cut-off: < = 2.55169) (n = 28)	High Phytohemagglutinin A (5 mg/L) group (Cut-off: > = 3.35315) (n = 28)	P value
Phytohemagglutinin A (5 mg/L) cpm / 1000 T cells	169.82 (100.0 - 288.4)[b]	3548.13 (2454.71 - 5128.61)	**< 0.0001**
Age	77.75 ± 7.86[c]	75.79 ± 7.05	NS
Nutrition variables			
Body mass index (kg/m^2)	26.34 ± 4.9	26.3 ± 6.87	NS
Protein (g/L)	69.1 ± 4.3	68.3 ± 7.0	NS
Albumin (g/L)	42.1 ± 2.5	40.9 ± 4.2	0.1983
Serum ferritin (μg/L)	47.86 (14.12 - 162.18)	69.18 (34.67 - 138.03)	0.1919
Serum transferrin receptor (mg/L)	6.6 ± 2.17	5.77 ± 1.73	0.1187
Transferrin saturation (%)	24.0 ± 8.54	25.04 ± 8.41	NS
Hemoglobin (g/L)	133.2 ± 8.1	138.3 ± 12.0	0.0656
Hematocrit (%)	40.01 ± 2.5	41.39 ± 3.62	0.1037
Mean cell volume (fL)	91.27 ± 4.87	93.75 ± 4.75	0.0586
Red cell distribution width (units)	13.19 ± 0.97	13.04 ± 1.1	NS
Plasma zinc (μg/L)	966.3 ± 169.1	931.1 ± 229.6	NS
Serum vitamin B_{12} (ng/L)	584.67 ± 349.2	504.99 ± 178.68	NS
Serum folate (μg/L)	18.82 ± 6.0	19.62 ± 12.25	NS

[a]Groups are defined based on percentiles (low ≤ 25th and high ≥ 75th) of log of Phytohemagglutinin A (5 mg/L) cpm / 1000 T cells
[b]Values are geometric mean and values in parentheses indicate ± 1 SD of geometric mean
[c]Values are mean ± SD

Table 2-7. Descriptive and nutrition variables by phytohemagglutinin A (10 mg/L) group

Variables	**Low Phytohemagglutinin A (10 mg/L) group[a] (Cut-off: < = 2.9367) (n = 30)**	**High Phytohemagglutinin A (10 mg/L) group (Cut-off: > = 3.53486) (n = 30)**	**P value**
Phytohemagglutinin A (10 mg/L) cpm / 1000 T cells	407.38 (169.82 - 977.23)[b]	4468.83 (3311.31 - 6025.59)	**< 0.0001**
Age	78.63 ± 7.86[c]	76.53 ± 7.06	NS
Nutrition variables			
Body mass index (kg/m^2)	26.51 ± 5.64	25.5 ± 6.65	NS
Protein (g/L)	69.3 ± 3.7	67.7 ± 5.8	NS
Albumin (g/L)	42.1 ± 2.6	41.0 ± 4.2	NS
Serum ferritin (µg/L)	56.23 (15.49 - 204.17)	72.44 (35.48 - 147.91)	NS
Serum transferrin receptor (mg/L)	5.65 ± 2.08	5.65 ± 1.35	NS
Transferrin saturation (%)	27.4 ± 10.69	27.2 ± 6.85	NS
Hemoglobin (g/L)	135.1 ± 8.3	139.0 ± 12.9	0.161
Hematocrit (%)	40.53 ± 2.52	41.65 ± 4.11	NS
Mean cell volume (fL)	91.28 ± 4.4	93.97 ± 5.06	**0.0323**
Red cell distribution width (units)	13.0 ± 1.0	13.02 ± 1.04	NS
Plasma zinc (µg/L)	946.8 ± 183.3	929.6 ± 220.0	NS
Serum vitamin B_{12} (ng/L)	574.09 ± 307.74	516.95 ± 163.25	NS
Serum folate (µg/L)	19.75 ± 7.51	19.04 ± 11.89	NS

[a]Groups are defined based on percentiles (low ≤ 25th and high ≥ 75th) of log of Phytohemagglutinin A (10 mg/L) cpm / 1000 T cells
[b]Values are geometric mean and values in parentheses indicate ± 1 SD of geometric mean
[c]Values are mean ± SD

Table 2-8. Descriptive and nutrition variables by concanavalin A (12 mg/L) group

Variables	**Low Concanavalin A (12 mg/L) group[a] (Cut-off: < = 2.70939) (n = 29)**	**High Concanavalin A (12 mg/L) group (Cut-off: > = 3.22286) (n = 29)**	**P value**
Concanavalin A (12 mg/L) cpm / 1000 T cells	204.17 (102.32 - 407.38)[b]	2754.23 (2041.73 - 3715.35)	**< 0.0001**
Age	77.41 ± 8.24[c]	76.03 ± 7.42	NS
Nutrition variables			
Body mass index (kg/m^2)	26.81 ± 6.52	24.56 ± 4.68	0.1363
Protein (g/L)	69.2 ± 3.1	68.0 ± 6.8	NS
Albumin (g/L)	42.4 ± 2.7	41.4 ± 3.9	NS
Serum ferritin (µg/L)	64.56 (29.51 - 141.25)	69.18 (28.84 - 165.95)	NS
Serum transferrin receptor (mg/L)	6.45 ± 2.17	6.21 ± 1.94	NS
Transferrin saturation (%)	24.9 ± 8.73	24.24 ± 8.37	NS
Hemoglobin (g/L)	134.6 ± 7.7	135.0 ± 14.1	NS
Hematocrit (%)	40.51 ± 2.22	40.71 ± 4.26	NS
Mean cell volume (fL)	89.57 ± 6.1	93.07 ± 5.26	**0.0229**
Red cell distribution width (units)	13.13 ± 0.9	13.38 ± 1.18	NS
Plasma zinc (µg/L)	925.2 ± 178.0	923.0 ± 221.1	NS
Serum vitamin B_{12} (ng/L)	545.83 ± 326.76	518.67 ± 174.81	NS
Serum folate (µg/L)	16.91 ± 6.35	17.85 ± 11.87	NS

[a]Groups are defined based on percentiles (low ≤ 25th and high ≥ 75th) of log of Concanavalin A (12 mg/L) cpm / 1000 T cells
[b]Values are geometric mean and values in parentheses indicate ± 1 SD of geometric mean
[c]Values are mean ± SD

Table 2-9. Descriptive and nutrition variables by concanavalin A (25 mg/L) group

Variables	**Low Concanavalin A (25 mg/L) group[a] (Cut-off: < = 2.82351) (n = 30)**	**High Concanavalin A (25 mg/L) group (Cut-off: > = 3.2579) (n = 30)**	**P value**
Concanavalin A (25 mg/L) cpm / 1000 T cells	275.42 (125.89 - 602.56)[b]	2691.53 (2137.96 - 3388.44)	**< 0.0001**
Age	76.47 ± 8.26[c]	76.47 ± 7.4	NS
Nutrition variables			
Body mass index (kg/m^2)	27.4 ± 6.37	24.16 ± 4.81	**0.0302**
Protein (g/L)	68.2 ± 4.0	68.5 ± 6.7	NS
Albumin (g/L)	41.5 ± 3.0	42.0 ± 4.1	NS
Serum ferritin (µg/L)	50.11 (20.89 - 120.23)	58.88 (22.39 - 154.88)	NS
Serum transferrin receptor (mg/L)	6.84 ± 2.64	6.46 ± 2.53	NS
Transferrin saturation (%)	25.13 ± 9.13	24.4 ± 10.19	NS
Hemoglobin (g/L)	134.2 ± 12.0	135.7 ± 12.9	NS
Hematocrit (%)	40.34 ± 3.66	40.86 ± 3.95	NS
Mean cell volume (fL)	90.95 ± 4.45	92.3 ± 5.12	NS
Red cell distribution width (units)	13.02 ± 0.97	13.3 ± 1.15	NS
Plasma zinc (µg/L)	837.9 ± 145.4	984.4 ± 240.2	**0.0412**
Serum vitamin B_{12} (ng/L)	548.62 ± 318.51	537.98 ± 164.42	NS
Serum folate (µg/L)	18.45 ± 7.87	17.78 ± 10.28	NS

[a]Groups are defined based on percentiles (low ≤ 25th and high ≥ 75th) of log of Concanavalin A (25 mg/L) cpm / 1000 T cells
[b]Values are geometric mean and values in parentheses indicate ± 1 SD of geometric mean
[c]Values are mean ± SD

Table 2-10. Discriminant Analysis Final Model for Classification of Individuals into Low and High Responders for Lymphocyte Proliferation Response to Phytohemagglutinin A and Concanavalin A

Final models	Variables selected in the model	Misclassification rate (%)
Phytohemagglutinin A (5 mg/L) cpm / 1000 T cells	Protein Serum ferritin Hemoglobin Hematocrit Red cell distribution width	15.4
Phytohemagglutinin A (10 mg/L) cpm / 1000 T cells	Protein Serum transferrin receptor Hemoglobin Hematocrit Mean corpuscular volume	20.7
Concanavalin A (12 mg/L) cpm / 1000 T cells	Protein Serum ferritin Hemoglobin Hematocrit Plasma zinc	10.3
Concanavalin A (25 mg/L) cpm / 1000 T cells	Protein Serum ferritin Hemoglobin Plasma zinc	15

Table 2-11. Descriptive and nutrition variables by IL-2 pg / 1000 total T cells group

Variables	**Low (IL-2 pg / 1000 total T cells) group[a] (Cut-off: < = -0.847322) (n = 21)**	**High (IL-2 pg / 1000 total T cells) group (Cut-off: > = -0.105416) (n = 21)**	**P value**
IL-2 pg / 1000 total T cells	0.09 (0.06 - 0.13)[b]	2.14 (1.00 - 4.57)	**< 0.0001**
Age	74.76 ± 6.98[c]	73.62 ± 7.39	NS
Nutrition variables			
Body mass index (kg/m^2)	24.93 ± 5.34	24.71 ± 7.07	NS
Protein (g/L)	68.7 ± 4.2	68.8 ± 4.0	NS
Albumin (g/L)	42.3 ± 2.3	40.4 ± 4.1	0.0618
Serum ferritin (µg/L)	26.9 (5.01 - 144.54)	60.25 (25.19 - 144.54)	0.0589
Serum transferrin receptor (mg/L)	7.07 ± 1.97	5.87 ± 1.9	0.0512
Transferrin saturation (%)	21.86 ± 9.86	27.19 ± 8.39	0.0662
Hemoglobin (g/L)	133.2 ± 10.1	140.4 ± 11.6	**0.0378**
Hematocrit (%)	40.06 ± 3.0	41.68 ± 3.61	0.1212
Mean cell volume (fL)	91.07 ± 5.78	91.19 ± 7.34	NS
Red cell distribution width (units)	13.11 ± 1.17	12.94 ± 0.94	NS
Plasma zinc (µg/L)	1115.5 ± 341.8	867.2 ± 115.1	**0.0075**
Serum vitamin B_{12} (ng/L)	754.78 ± 457.21	451.26 ± 142.91	**0.0061**
Serum folate (µg/L)	18.62 ± 4.91	19.59 ± 12.7	NS

[a]Groups are defined based on percentiles (low ≤ 25th and high ≥ 75th) of log of IL-2 pg / 1000 total T cells
[b]Values are geometric mean and values in parentheses indicate ± 1 SD of geometric mean
[c]Values are mean ± SD

Table 2-12. Descriptive and nutrition variables by IL-2 pg / 1000 T helper cells group

Variables	**Low (IL-2 pg / 1000 T helper cells) group[a] (Cut-off: < = -0.7155145) (n = 21)**	**High (IL-2 pg / 1000 T helper cells) group (Cut-off: > = 0.067613) (n = 21)**	**P value**
IL-2 pg / 1000 T helper cells	0.14 (0.11 - 0.18)[b]	3.31 (1.44 - 7.58)	**< 0.0001**
Age	75.24 ± 7.28[c]	73.9 ± 7.48	NS
Nutrition variables			
Body mass index (kg/m^2)	25.33 ± 4.95	25.67 ± 7.12	NS
Protein (g/L)	70.0 ± 6.2	67.7 ± 3.4	0.1396
Albumin (g/L)	42.3 ± 2.3	39.4 ± 3.5	**0.0024**
Serum ferritin (μg/L)	33.11 (5.62 - 194.98)	54.95 (21.87 - 138.04)	NS
Serum transferrin receptor (mg/L)	6.97 ± 2.12	6.24 ± 2.72	NS
Transferrin saturation (%)	22.86 ± 10.61	26.1 ± 8.88	NS
Hemoglobin (g/L)	131.0 ± 9.9	140.3 ± 9.9	**0.0044**
Hematocrit (%)	39.35 ± 2.91	41.8 ± 2.95	**0.0099**
Mean cell volume (fL)	90.83 ± 5.76	91.62 ± 7.29	NS
Red cell distribution width (units)	13.07 ± 1.2	12.93 ± 0.93	NS
Plasma zinc (μg/L)	1092.8 ± 492.4	857.5 ± 130.4	0.0617
Serum vitamin B_{12} (ng/L)	731.01 ± 450.41	464.6 ± 145.63	**0.014**
Serum folate (μg/L)	19.22 ± 3.48	20.04 ± 12.37	NS

[a]Groups are defined based on percentiles (low ≤ 25th and high ≥ 75th) of log of IL-2 pg / 1000 T helper cells
[b]Values are geometric mean and values in parentheses indicate ± 1 SD of geometric mean
[c]Values are mean ± SD

Table 2-13. Descriptive and nutrition variables by IL-1 pg / 1000 granulocytes group

Variables	**Low (IL-1 pg / 1000 granulocytes) group**[a] (Cut-off: < = -0.5080914) **(n = 26)**	**High (IL-1 pg / 1000 granulocytes) group (Cut-off: > = 0.0196512) (n = 25)**	**P value**
IL-1 pg / 1000 granulocytes	0.18 (0.09 - 0.34)[b]	1.55 (1.15 - 2.09)	**< 0.0001**
Age	76.65 ± 7.17[c]	77.88 ± 6.35	NS
Nutrition variables			
Body mass index (kg/m^2)	27.2 ± 6.84	26.0 ± 5.38	NS
Protein (g/L)	67.7 ± 6.2	68.9 ± 5.6	NS
Albumin (g/L)	41.2 ± 3.8	42.3 ± 3.5	NS
Serum ferritin (µg/L)	52.48 (21.38 - 128.82)	66.07 (24.54 - 177.82)	NS
Serum transferrin receptor (mg/L)	6.83 ± 2.31	6.20 ± 2.01	NS
Transferrin saturation (%)	23.38 ± 8.28	26.48 ± 8.74	NS
Hemoglobin (g/L)	138.7 ± 15.1	137.5 ± 10.8	NS
Hematocrit (%)	41.8 ± 4.69	41.29 ± 3.35	NS
Mean cell volume (fL)	91.06 ± 3.29	91.34 ± 6.98	NS
Red cell distribution width (units)	13.2 ± 1.11	13.05 ± 0.83	NS
Plasma zinc (µg/L)	954.3 ± 206.6	909.8 ± 161.2	NS
Serum vitamin B_{12} (ng/L)	482.61 ± 163.02	618.86 ± 299.7	**0.0481**
Serum folate (µg/L)	15.6 ± 6.86	16.76 ± 6.94	NS

[a]Groups are defined based on percentiles (low ≤ 25th and high ≥ 75th) of log of IL-1 pg / 1000 granulocytes
[b]Values are geometric mean and values in parentheses indicate ± 1 SD of geometric mean
[c]Values are mean ± SD

Table 2-14. Descriptive and nutrition variables by IL-1 pg / 1000 granulocytes and monocytes group

Variables	**Low (IL-1 pg / 1000 granulocytes and monocytes) group[a] (Cut-off: < = -0.5344203) (n = 26)**	**High (IL-1 pg / 1000 granulocytes and monocytes) group (Cut-off: > = -0.035866) (n = 25)**	**P value**
IL-1 pg / 1000 granulocytes and monocytes	0.17 (0.09 - 0.32)[b]	1.38 (1.02 - 1.86)	**< 0.0001**
Age	75.54 ± 7.13[c]	77.88 ± 6.35	NS
Nutrition variables			
Body mass index (kg/m^2)	26.92 ± 6.79	26.0 ± 5.38	NS
Protein (g/L)	67.7 ± 6.3	68.9 ± 5.6	NS
Albumin (g/L)	41.2 ± 3.8	42.3 ± 3.5	NS
Serum ferritin (µg/L)	51.28 (21.87 - 120.22)	66.07 (24.54 - 177.82)	NS
Serum transferrin receptor (mg/L)	6.88 ± 2.28	6.2 ± 2.01	NS
Transferrin saturation (%)	22.85 ± 7.2	26.48 ± 8.74	0.1111
Hemoglobin (g/L)	140.1 ± 15.1	137.5 ± 10.8	NS
Hematocrit (%)	42.18 ± 4.69	41.29 ± 3.35	NS
Mean cell volume (fL)	91.06 ± 3.29	91.34 ± 6.98	NS
Red cell distribution width (units)	13.17 ± 1.14	13.05 ± 0.83	NS
Plasma zinc (µg/L)	962.9 ± 207.9	909.8 ± 161.2	NS
Serum vitamin B_{12} (ng/L)	484.51 ± 165.12	618.86 ± 299.7	0.0518
Serum folate (µg/L)	15.43 ± 6.82	16.76 ± 6.94	NS

[a]Groups are defined based on percentiles (low ≤ 25th and high ≥ 75th) of log of IL-1 pg / 1000 granulocytes and monocytes
[b]Values are geometric mean and values in parentheses indicate ± 1 SD of geometric mean
[c]Values are mean ± SD

Table 2-15. Descriptive and nutrition variables by IL-6 pg / 1000 granulocytes group

Variables	**Low (IL-6 pg / 1000 granulocytes) group[a] (Cut-off: < = 0.9408286) (n = 29)**	**High (IL-6 pg / 1000 granulocytes) group (Cut-off: > = 1.2940519) (n = 29)**	**P value**
IL-6 pg / 1000 granulocytes	4.78 (2.82 - 8.13)[b]	28.18 (20.14 - 38.9)	**< 0.0001**
Age	76.52 ± 7.1[c]	76.79 ± 7.61	NS
Nutrition variables			
Body mass index (kg/m^2)	25.65 ± 6.45	24.8 ± 5.23	NS
Protein (g/L)	68.1 ± 5.0	69.1 ± 5.4	NS
Albumin (g/L)	40.4 ± 3.3	43.1 ± 2.6	**0.0019**
Serum ferritin (µg/L)	51.28 (14.79 - 177.82)	52.48 (14.12 - 194.98)	NS
Serum transferrin receptor (mg/L)	6.53 ± 2.14	5.99 ± 2.09	NS
Transferrin saturation (%)	22.21 ± 7.11	26.97 ± 10.63	**0.0451**
Hemoglobin (g/L)	134.8 ± 13.2	135.5 ± 9.3	NS
Hematocrit (%)	40.46 ± 3.89	40.83 ± 2.99	NS
Mean cell volume (fL)	90.07 ± 5.55	92.31 ± 5.47	0.0595
Red cell distribution width (units)	13.32 ± 1.28	12.98 ± 0.92	NS
Plasma zinc (µg/L)	942.2 ± 202.2	938.9 ± 166.8	NS
Serum vitamin B_{12} (ng/L)	559.76 ± 245.12	643.27 ± 372.44	NS
Serum folate (µg/L)	19.26 ± 11.56	18.55 ± 4.5	NS

[a]Groups are defined based on percentiles (low ≤ 25th and high ≥ 75th) of log of IL-6 pg / 1000 granulocytes
[b]Values are geometric mean and values in parentheses indicate ± 1 SD of geometric mean
[c]Values are mean ± SD

Table 2-16. Descriptive and nutrition variables by IL-6 pg / 1000 granulocytes and monocytes group

Variables	**Low (IL-6 pg / 1000 granulocytes and monocytes) group[a] (Cut-off: < = 0.9069067) (n = 30)**	**High (IL-6 pg / 1000 granulocytes and monocytes) group (Cut-off: > = 1.262338) (n = 30)**	**P value**
IL-6 pg / 1000 granulocytes and monocytes	5.24 (3.09 - 8.91)[b]	30.9 (22.38 - 42.65)	**< 0.0001**
Age	76.7 ± 7.05[c]	77.57 ± 7.39	NS
Nutrition variables			
Body mass index (kg/m^2)	25.57 ± 6.35	24.49 ± 5.14	NS
Protein (g/L)	68.2 ± 5.0	69.2 ± 5.2	NS
Albumin (g/L)	40.6 ± 3.4	43.1 ± 2.5	**0.0012**
Serum ferritin (µg/L)	52.48 (15.48 - 177.82)	53.7 (14.79 - 194.98)	NS
Serum transferrin receptor (mg/L)	6.53 ± 2.1	5.93 ± 2.09	NS
Transferrin saturation (%)	22.3 ± 7.0	27.0 ± 10.44	**0.0499**
Hemoglobin (g/L)	135.1 ± 13.1	134.9 ± 9.1	NS
Hematocrit (%)	40.56 ± 3.85	40.68 ± 2.94	NS
Mean cell volume (fL)	90.02 ± 5.46	92.67 ± 5.21	0.1217
Red cell distribution width (units)	13.27 ± 1.28	13.0 ± 0.9	NS
Plasma zinc (µg/L)	942.2 ± 202.2	907.7 ± 165.8	NS
Serum vitamin B_{12} (ng/L)	563.2 ± 241.59	655.94 ± 355.73	NS
Serum folate (µg/L)	19.15 ± 11.38	19.39 ± 5.33	NS

[a]Groups are defined based on percentiles (low ≤ 25th and high ≥ 75th) of log of IL-6 pg / 1000 granulocytes and monocytes
[b]Values are geometric mean and values in parentheses indicate ± 1 SD of geometric mean
[c]Values are mean ± SD

Table 2-17. Discriminant analysis final model for classification of individuals into low and high responders for various cytokines

Final models	Variables selected in the model	Misclassification rate (%)
IL-2 pg / 1000 T cells	Protein Serum ferritin Serum vitamin B_{12}	13.4
IL-2 pg / 1000 T helper cells	Protein Serum ferritin Serum vitamin B_{12}	11.1
IL-1 pg / 1000 granulocytes	Protein Serum transferrin receptor Hematocrit Serum vitamin B_{12} Serum folate	22.2
IL-1 pg / 1000 granulocytes and monocytes	Protein Serum transferrin receptor Hematocrit Serum vitamin B_{12} Serum folate	19.5
IL-6 pg / 1000 granulocytes	Protein Serum transferrin receptor Hematocrit Serum vitamin B_{12} Serum folate	18.9
IL-6 pg / 1000 granulocytes and monocytes	Protein Serum transferrin receptor Hematocrit Serum vitamin B_{12} Serum folate	17.5

Table 2-18. Summary of discriminant analysis final models for classification of individuals into low and high responders on various immune function tests

Variables*	BMI	Protein	Alb	Serum Ft	Serum TfR	TS	Hb	Hct	MCV	RDW	Plasma zinc	Serum vitamin B_{12}	Serum folate	Error rate (%)
T cells and subsets														
Total T cells		**x**		**x**					**x**		**x**			37.2
T helper cells	**x**	**x**					**x**	**x**			**x**			16.5
T cytotoxic cells			**x**				**x**		**x**		**x**			25.4
Mitogen proliferation														
PHA 5/1000 T cells		**x**		**x**			**x**	**x**		**x**				15.4
PHA 10/1000 T cells		**x**			**x**		**x**	**x**	**x**					20.7
Con A 12/1000 T cells		**x**		**x**			**x**	**x**			**x**			10.3
Con A 25/1000 T cells		**x**		**x**			**x**				**x**			15
Cytokines														
IL-2/1000 T cells		**x**		**x**								**x**		13.4
IL-2/1000 T helper cells		**x**		**x**								**x**		11.1
IL-1/1000 granulocytes		**x**			**x**			**x**				**x**	**x**	22.2
IL-1/1000 granulocytes and monocytes		**x**			**x**			**x**				**x**	**x**	19.5
IL-6/1000 granulocytes		**x**			**x**			**x**				**x**	**x**	18.9
IL-6/1000 granulocytes and monocytes		**x**			**x**			**x**				**x**	**x**	17.5

*Alb – albumin; Ft – ferritin; TfR – transferrin receptor; TS – transferrin saturation; Hb – hemoglobin; Hct – hematocrit; RDW – red cell distribution width

Table 2-19. Correlation between nutrition and immune function variables[a]

	CD3	CD4	CD8	PH5	PH10	CN12	CN25	IL2KT	IL2KTH	IL1KG	IL1KMG	IL6KG	IL6KMG
BMI	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Protein	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Albumin	NS	NS	NS	NS	NS	NS	NS	-0.26 0.015 85	-0.31 0.004 84	NS	NS	0.29 0.001 120	0.29 0.001 118
Lsft	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
STfR	NS	NS	NS	NS	NS	NS	NS	-0.23 0.037 85	-0.22 0.044 84	NS	NS	NS	NS
TS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	0.24 0.008 120	0.24 0.009 118
Hb	NS	NS	NS	0.22 0.024 112	NS	NS	NS	0.25 0.023 85	0.25 0.02 85	NS	NS	NS	NS
Hct	NS	NS	NS	0.19 0.04 112	NS	NS	NS	NS	NS	NS	NS	NS	NS
MCV	NS	NS	NS	NS	NS	0.22 0.016 118	NS	NS	NS	NS	NS	0.24 0.009 120	0.23 0.009 118
RDW	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	-0.19 0.034 120	-0.18 0.05 118
Zinc	NS	NS	NS	NS	NS	NS	0.29 0.013 74	-0.38 0.003 60	-0.39 0.002 59	NS	NS	NS	NS
Vit B_{12}	NS	NS	NS	NS	NS	NS	NS	-0.32 0.004 82	-0.31 0.005 81	NS	NS	NS	NS
Folate	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

[a]Each box of the matrix represents Pearson correlation coefficients, p values and number of observations; NS – no significant relationship

Table 2-20. Immune function variables by body mass index

Variables	Normal group (BMI > 18.5 and < 25) (n = 63)	Overweight group (BMI >= 25 and < 30) (n = 26)	Obese group (BMI >= 30) (n = 29)	P value
T cells and subsets (absolute numbers)				
Total T cells (x 10^9/L)	1.36 ± 0.53[a]	1.58 ± 0.91	1.47 ± 0.74	NS
T helper cells (x 10^9/L)	0.9 ± 0.36	1.03 ± 0.59	0.97 ± 0.49	NS
T cytotoxic cells (x 10^9/L)	0.45 ± 0.28	0.49 ± 0.34	0.53 ± 0.44	NS
Lymphocyte proliferation response				
Phytohemagglutinin A (5 mg/L) cpm / 1000 T cells	933 (288 - 3020)[b]	616 (182 - 2089)	891 (234 - 3388)	NS
Phytohemagglutinin A (10 mg/L) cpm / 1000 T cells	1622 (513 - 5129)	1148 (417 - 3162)	1621 (676 - 3890)	NS
Concanavalin A (12 mg/L) cpm / 1000 T cells	912 (309 - 2691)	676 (219 - 2089)	832 (324 - 2138)	NS
Concanavalin A (25 mg/L) cpm / 1000 T cells	1047 (398 - 2754)	794 (263 - 2399)	891 (398 - 1995)	NS
Cytokine production				
IL-2 pg / 1000 T cells	0.39 (0.11 - 1.05)	0.33 (0.08 - 1.41)	0.46 (0.11 - 1.86)	NS
IL-2 pg / 1000 T helper cells	0.52 (0.16 - 1.7)	0.5 (0.12 - 2.19)	0.69 (0.19 - 2.45)	NS
IL-1 pg / 1000 granulocytes	0.59 (0.25 - 1.41)	0.65 (0.31 - 1.35)	0.47 (0.17 - 1.26)	NS
IL-1 pg / 1000 granulocytes and monocytes	0.53 (0.23 - 1.23)	0.57 (0.27 - 1.23)	0.44 (0.16 - 1.21)	NS
IL-6 pg / 1000 granulocytes	13.8 (6.61 - 28.84)	12.88 (6.76 - 24.55)	11.48 (5.25 - 25.12)	NS
IL-6 pg / 1000 granulocytes and monocytes	12.59 (6.03 - 26.3)	11.75 (6.03 - 22.91)	10.23 (4.68 - 22.39)	NS

[a]Values are mean ± SD
[b]Values are geometric mean and values in parentheses indicate ± 1 SD of geometric mean

2.4 Discussion

Nutrition is important for maintaining immune function in older adults (Chandra, 2002; High, 1999; Lesourd et al., 1998). Various studies have been conducted in older adults, focusing on single or multiple nutrients and/or the effect of supplementation of these nutrients on immune function (Bogden et al., 1994; Buzina-Suboticanec et al., 1998; Chandra, 1992; Goodwin and Garry, 1988; Lesourd 1995; Meydani et al., 1991; Meydani et al., 1990). However, very few studies have focused on comprehensively examining multiple nutrients at the same time and their effect on immune response (Bogden et al., 1994; Buzina-Suboticanec et al., 1998; Chandra, 1992; Fülöp et al., 1999; Goodwin and Garry, 1988; Goodwin and Garry, 1983; Payette et al., 1990). Most of these studies do not address the issue of whether nutrients can predict immune function outcomes. Therefore, the focus of our study was to examine the relationship between certain nutrients namely, protein, iron, zinc, vitamin B_{12} and folate simultaneously with tests of acquired immunity, such as T cells and subsets, lymphocyte proliferation response, and cytokine production. Our study was unique in that we employed multivariate analysis to determine relation between nutrients and immune function in a cohort of women with varied nutritional status. The specific objective of the study was to identify a subset of nutrients, which can classify healthy older women (> 60 years) as low or high responders on the tests of acquired immune function, by using discriminant analysis approach.

The nutrition variables included in the study were BMI, serum protein and albumin for protein status; serum ferritin, serum transferrin receptor, transferrin saturation, hemoglobin, hematocrit, mean corpuscular volume, and red cell distribution width for iron status; plasma zinc, serum vitamin B_{12} and serum folate. Acquired immune function variables, namely CD3+, CD4+ and CD8+ absolute numbers, lymphocyte proliferation response to PHA (5 and 10 mg/L) and Con A (12 and 25 mg/L), and cytokine production namely IL-2, IL-1 and IL-6 were measured

using whole blood assays as per previous studies in our laboratory (Krause et al., 1999). Whole blood assays are advantageous in that they are simple, reproducible, utilize small amounts of blood and they do not require separation of mononuclear cells or standardization of number of lymphocytes (Bloemena et al., 1989).

The statistical approach of discriminant analysis was advantageous over other multivariate analysis such as multiple regression or ANOVA as it provided a parsimonious predictive subset of nutrients, which classified healthy older women into low and high responders on various acquired immune response. Low responders had values for the immune variable ≤ 25th percentile of the study cohort, whereas, high responders had ≥ 75th percentile of the study cohort for the immune variable. This approach to define low and high responders, similar to the NMAP study of Goodwin and Garry (1988), was appropriate because if the nutrients were important predictors of immune tests, they would certainly appear between extreme groups of immune function; and also because there was sufficient power in the two extreme groups to detect relationships between nutrition and immune function. Further, discriminant analysis approach offered the ability to test the final predictive model in terms of its cross-validation, as it provided estimates of misclassification rates for classifying subjects using the final predictive model into the appropriate groups of high and low responders on the immune function variable. This kind of multivariate statistical analysis, involving multiple nutrients and immune function, has been investigated in very few studies (Goodwin and Garry, 1988; Goodwin and Garry, 1983; Payette et al., 1990). It is of importance to statistically approach the aspect of nutrition and immune function in this manner, as many nutrients can influence immune function (Beisel et al., 1981; High, 1999). In addition, studying multiple nutrients in older adults is valuable as multiple nutrient deficiencies can coexist in this group (Chandra, 2002; Tucker, 1995).

Contrary to previous findings involving multivariate analyses (Gardner et al., 2000; Goodwin and Garry, 1988; Goodwin and Garry, 1983; Payette et al., 1990), in our study nutrition

variables emerged as significant predictors of immune function. For T cells and subsets, protein, iron and zinc status variables emerged in the final predictive subsets. The predictive subset classified subjects with a high degree of accuracy; the probability of correctly classifying subjects was 62.8%, 83.5% and 74.6%, for CD3+, CD4+ and CD8+ groups respectively.

The relationship of protein, iron and zinc status with T cells and subsets has been illustrated by many studies. Lesourd (1995) demonstrated the effect of protein on T cells and subsets, by comparing two groups of older adults selected using the SENIEUR protocol, one group being well-nourished and the other being undernourished (with serum albumin between 30-35 g/L in the absence of acute phase reactions in the body). The two groups differed on CD3+ and CD4+ numbers, with the undernourished group having significantly lower cell numbers ($P<0.05$). Similar findings have been observed in children, where significantly reduced proportions of CD4+ ($P<0.001$) and CD8+ ($P<0.05$) cells were noted in malnourished versus well-nourished children (Chandra et al., 1982). Our findings are similar to these studies on protein, as our results also suggest that protein is important for T cells and subsets.

The literature presents mixed findings on the effect of iron status on T cells and subsets in adults and children (Bagchi et al., 1980; Berger et al., 1992; Thibault et al., 1993), however, limited data exist on iron status and immune function in older adults (Kamenetz et al., 1998), especially the healthy older adults (Ahluwalia et al., 2003). Ahluwalia and colleagues (2003) showed that iron-deficient and iron-sufficient homebound healthy older women, selected as per the SENIEUR protocol and other health criteria, did not differ on CD3+, CD4+ or CD8+ cell numbers. However, our results show that iron along with protein and zinc is important for number of T cells and subsets. Our findings may differ from those of Ahluwalia et al. (2003) as they had a strict subject selection criteria with SENIEUR protocol, in addition to controlling for some nutrient deficiencies such as protein, and vitamin B_{12}; whereas we included women free from inflammation who were selected using SENIEUR protocol criteria but having varying

nutritional status for iron, zinc, folate and vitamin B_{12}. The effect of zinc on T cells and subsets in older persons has also been demonstrated (Duchateau et al., 1981; Fortes et al., 1998). Fortes et al. (1998) showed that zinc treatment of 25 mg zinc sulfate significantly increased the number of CD4+ ($P=0.012$) and CD8+ ($P=0.005$) cell numbers in 118 healthy older home residents; these findings, similar to our study findings, suggest the importance of zinc for T cell subsets.

In our results, for lymphocyte proliferation response to PHA and Con A, protein and iron status variables emerged in the final predictive subsets; however zinc was identified in the final predictive subset for lymphocyte proliferation response to Con A only. The probability of correctly classifying subjects into high or low PHA variables were 84.6% and 79.3%; and for Con A variables the probability of correctly classifying subjects into high or low groups was higher with 89.7% and 85%.

Many studies document associations between protein, iron and zinc status with lymphocyte proliferation response to PHA and Con A. In Lesourd's (1995) study, comparisons between well-nourished and undernourished SENIEURS showed that protein status was important for lymphocyte proliferation response. Undernourished SENIEURS with low protein status had significantly lower lymphocyte proliferation response to PHA (0.25 $\mu g/10^6$ cells) than healthy well-nourished SENIEURS. The findings from Lesourd's study (1995) are consistent with our findings and indicate that protein is important for lymphocyte functions.

With respect to iron and lymphocyte proliferation response, many studies have documented the importance of iron for this immune function response (Ahluwalia et al., 2003; Macdougall et al., 1975; Thibault et al., 1993). Ahluwalia et al. (2003) showed that iron-deficient homebound women have significantly lower lymphocyte proliferation response to PHA ($P<0.05$) and Con A ($P<0.01$) than iron-sufficient women. Macdougall et al. (1975) noted similar findings in children, where anemic and iron-deficient children had significantly lower lymphocyte response to PHA than controls ($P<0.02$). The studies by Ahluwalia et al. (2003) and Macdougall

et al. (1975) are consistent with our results and show that iron is important for lymphocyte proliferation. In contrast to the present findings, one study found no difference in the proliferative response of lymphocytes to PHA of iron-deficient and iron-sufficient children (Thibault et al., 1993). The authors of this study speculate that this may be due to lack of exclusion of other associated nutritional deficiencies and the high prevalence of isolated depletion of iron stores (61%) in the children who were not iron-deficient.

The relationship between zinc and immune function is illustrated by a depletion-repletion study of 8 healthy men ranging from 27-47 years (Pinna et al., 2002). Subjects' immune responses were measured at baseline, after 10 weeks of zinc restriction, followed by zinc repletion for 5 weeks. During baseline and repletion, subjects zinc intake was 13.7 mg/day; whereas during depletion it was 4.6 mg/day. The results showed that with zinc restriction, proliferation of peripheral blood mononuclear cells to PHA (at concentrations of 1.2, 2.5, 5 and 20 mg/L) was significantly reduced ($P<0.01$) compared to baseline values; and this diminished proliferation was not restored by 5 weeks of repletion. The authors suggest that the time for zinc repletion may not have been sufficient to reverse the effects of low intake, and that the amount of time required to restore immune function is not known. However, their findings, similar to ours, suggest that zinc is needed for lymphocyte proliferation. Chandra (1991) also noted similar findings of decreased lymphocyte proliferation response to PHA in children with acrodermatitis enteropathica; and this decreased response was restored to normal after treatment with 150 mg zinc/day for 6 weeks. In another study, the effect of zinc on lymphocyte proliferation response was determined in a 1-year supplementation study of 63 apparently healthy older subjects (Bogden et al., 1990). Subjects were given placebo, 15 mg zinc or 100 mg zinc daily along with a zinc-free multinutrient supplement. Lymphocyte proliferation response to PHA but not Con A improved over the course of the study, especially in the placebo group. These findings are in contrast to our study as we showed that zinc was an important predictive variable for lymphocyte

proliferation response to Con A and not PHA. An interesting speculation by Bogden and colleagues (1990) regarding their study findings is that the improvements noted in lymphocyte proliferation response to PHA, especially with placebo, may have been due to the multinutrient supplement that was also provided to subjects, and not just zinc alone.

In the last section of our results, we present findings regarding which nutrients were in the predictive subsets for IL-1, IL-2 and IL-6 variables. For all cytokine variables, protein, iron and vitamin B_{12} status variables emerged as predictors in the final subsets, and folate was identified for IL-6 variables only. The probability of correctly classifying subjects into high or low responders on various cytokine groups was high and ranged from 77.8-88.9%.

Few studies have observed the relationship between these nutrients, namely protein, iron and vitamin B_{12} and folate with cytokine production in older adults (Kamenetz et al., 1998; Lesourd, 1995). The effect of protein and folate status on cytokine production in older adults, selected by SENIEUR protocol, is illustrated in studies by Lesourd's group; and their results are similar to our findings suggesting that protein and folate are vital for cytokine production (Lesourd, 1995; Lesourd and Mazari, 1999; Mazari and Lesourd, 1998). In one study, well-nourished older subjects had significantly lower IL-2 synthesis ($P<0.05$) from PHA stimulated cultures as compared to undernourished older subjects who had low serum albumin levels in the absence of acute phase reactions (Lesourd, 1995). In another study, Lesourd and Mazari (1999) observed significantly lower IL-6 ($P<0.01$) and a trend of lower IL-1 levels from lipopolysachharide-stimulated monocytes cultures of older subjects with PEM compared to healthy older subjects. Similar findings were found with respect to folate by Mazari and Lesourd (1998) who compared SENIEURS with low folate versus healthy SENIEURS on IL-2 and IL-6 production. Their results showed that SENIEURS with low folate expressed significantly lower IL-2 synthesis ($P<0.05$) and a trend of low IL-6 synthesis compared to healthy older subjects. The studies on vitamin B_{12} status and cytokines are limited. However, one study by Peracchi et

al. (2001) investigated the levels of serum tumor necrosis factor (TNF)-α, a proinflammatory cytokine like IL-1 and IL-6, in 34 adult patients with cobalamin deficiency. They found that TNF-α was significantly higher in patients with cobalamin deficiency ($P<0.01$), and was normalized after cobalamin treatment. These findings, similar to ours, suggest the role of these proinflammatory cytokines for vitamin B_{12} status. In another study, Funada et al (2001) showed that interferon (IFN)-γ, a T-helper 1 or CD4+ cytokine just like IL-2, was significantly lower in vitamin B_{12} deficient mice than control mice; these findings are similar to ours as they suggest the relation between CD4+ cytokines like IL-2 and vitamin B_{12}.

Most of the studies on iron status and cytokine production have been in children (Bhaskaram et al., 1989; Sipahi et al., 1998; Thibault et al., 1993). These studies suggest that IL-2 levels are lower than normal levels, whereas pro-inflammatory cytokines, IL-1 and IL-6 remain unchanged in iron-deficiency. Kamenetz et al. (1998) compared serum IL-6 levels in 17 older subjects with unexplained mild anemia (defined as hemoglobin < 120 g/L and without iron deficiency, anemia of chronic disease, megaloblastic anemia or any other hematologic disorder) to 14 healthy older subjects. Although, serum IL-6 levels were lower in the anemic group versus control group, this finding was not statistically significant. Nevertheless, these findings are consistent with ours in that they suggest that iron status is important for IL-6 production.

We related our study results to previous findings of nutrition and immune function, where multiple nutrients were simultaneously determined or further even statistically analyzed at the same time with immune function tests (Chandra, 1992; Gardner et al., 2000; Goodwin and Garry, 1988; Payette et al., 1990). Chandra (1992) supplemented healthy older adults (> 65 years) daily for a year with either placebo (n=48) or with a micronutrient formulation containing low doses of nine vitamins, five trace elements and higher levels of antioxidants vitamins C, E, and β-carotene (n=48). At baseline, prevalence of nutrient deficiencies did not differ between the two groups, however, with supplementation, statistically significant reduction in deficiencies of vitamin A, β-

carotene, vitamin B_6, vitamin C, iron and zinc were noted. Immunological tests, including number of T cells, lymphocyte proliferation to PHA, IL-2 production, and IL-2R release improved ($P>0.05$) with supplementation. In addition, a significant correlation was noted between serum zinc and IL-2 production. Although our study was not an intervention, the findings are consistent with Chandra's study in that they suggest the importance of these nutrients for T cell functions in the study.

Goodwin and Garry (1988) conducted a large study with 230 independently living healthy older men and women of the NMAP study cohort. Information on intake and status of several nutrients, albumin, vitamins A, B_{12}, C, D, E, folate, riboflavin, copper, iron and zinc, and measurements of several immune function tests, DTH skin tests, lymphocyte proliferation response to PHA, lymphocyte counts, and serum autoantibodies and circulating immune complexes were obtained. Simple correlation or logistic regressions between blood levels of various nutrients and measurements of immune status did not show any significant associations. The authors then took a unique approach by comparing the immune responses of individuals with evidence of inadequate nutrition to individuals with normal nutrition i.e. they compared the results of immunologic tests for subjects in the bottom 5%, bottom 10% and top 90% with respect to blood levels of specific nutrients. No significant associations were found between malnutrition and depressed immunologic functions. Therefore, these study findings are different from our results, as they do not indicate that none of the nutrients studied were associated with immune function tests. Although the large size of the NMAP population was ensured so that true associations were not missed, the study was conducted with an affluent well-educated group where the range of nutrient intakes and status may not have been wide.

Another study examined the relationship between nutrition and immunologic status by regression analysis in 82 healthy free-living older adults (Payette et al., 1990). The predictive variables included anthropometric and dietary data, blood levels of eight nutrients, hematologic

values and plasma fatty acids; and the immune variables included cytotoxicity of natural killer (NK) cells and activity of IL-2. None of the nutrition factors was significantly correlated with cytotoxic activity of NK cells ($P<0.05$); however, the dietary intakes of vitamins E and D were negatively associated with the activity of IL-2 ($P<0.007$). Although no association was found between most nutrients and immune function tests, the authors suggest that an association may at least exist between particular aspects of nutrition status and regulation of immune response by IL-2. In addition, the study cohort comprised of healthy, non-institutionalized individuals who may not have had nutrient deficiencies. Unfortunately, we cannot relate our findings to this study, as Payette and colleagues did not measure other immune function tests such as those included in our study. In another study, Gardner et al. (2000) investigated immune responses and plasma concentrations of β-carotene, retinol, α-tocopherol and zinc of 61 healthy old and 27 young adults before and after immunization with influenza vaccine. Plasma concentrations of retinol and zinc did not change pre- and post-immunization in both groups, however, β-carotene and α-tocopherol were significantly higher pre- to post-immunization in the old ($P \leq 0.004$), but not in the young. No correlations were found between micronutrients at pre-immunization and proliferative or antibody responses to any of the viral components post-immunization. Therefore, the authors concluded that even when plasma micronutrients are maintained in older adults, there is still an impaired immune response to influenza vaccine. Further, the authors categorized individuals as having intact antibody response if there was fourfold rise in post-vaccination titer and produced post-vaccination titers ≥ 40 to at least one vaccine component; and they found that 24.6% of older adults versus 70.4% of young had intact antibody responses. Importantly, plasma micronutrient levels were comparable for older adults with or without intact antibody responses after vaccination. In addition, no correlations were found between plasma micronutrients (at pre- or post-immunization) and percentage of older adults producing intact antibody responses to the vaccine. In contrast to our study findings that nutrients are important for immune response, these

results suggest that differences in these plasma micronutrients are not are not associated with differences in antibody responses among healthy older adults. It is important to note that Gardner and colleagues (2000) did not measure protein status, which could have confounded antibody responses.

In our study cohort, as part of secondary analysis, we compared groups of women with varying BMI status on various immune function variables (Table 2-20). The three groups included normal women with BMI > 18.5 and < 25, overweight women with BMI >= 25 and < 30, and obese women with BMI >= 30. None of the groups differed significantly on any of the immune function variables. This implies that in our study cohort, obesity is not associated with immune function. It is possible that this association may not have been captured because our study cohort tended to be lean, relative to the general older population, due to the fact that women with chronic diseases were excluded. It is likely that women with chronic diseases such as heart disease or rheumatoid arthritis may be more obese than a generally healthy cohort, thus resulting in a leaner cohort in our study.

A summary of our results from the final discriminant models for each immune function variable shows that protein and iron status variables were identified in the predictive subset for all immune function variables examined; zinc emerged in the final predictive subset for T cells and subsets, and lymphocyte proliferation response to Con A. Interestingly, vitamin B_{12} and folate were identified in the final predictive subset only for cytokine variables. Studies have put forth mechanisms by which protein, iron, zinc and vitamin B_{12} have been known to influence immunocompetence. It has been suggested that the immunologic responses in PEM such as low antibody synthesis or low antigen-derived clonal expansion of lymphocytes is brought about by the endocrine hormonal system that governs the rate and direction of flow of substances and energy (Woodward, 1998). Iron is important for iron-dependant enzymes such as ribonucleotide reductase; and the activation of protein kinase C and hydrolysis of cell-membrane phospholipids

important for signal transduction, and thereby leading to T cell proliferation and other functions (Kuvibidila and Baliga, 2002). Much evidence exists for the mechanism by which zinc affects immune cells (Shankar and Prasad, 1998). Zinc influences the activity of several enzymes important for replication and transcription, such as DNA polymerase and thymidine kinase. It is crucial for lymphocyte activation as it is needed by phospholipase C and also affects phosphorylation of protein kinase C; zinc is a major intracellular regulator of lymphocyte apoptosis (Fraker and King, 2001). Zinc is also needed for immune mediators like thymulin (Shankar and Prasad, 1998). Vitamin B_{12} can influence lymphocyte proliferation as it is needed for cell growth and division, as it serves as a coenzyme for DNA/nucleic acid synthesis (Myrvik, 1999).

The present study was a carefully controlled examination of the relationship between nutrition and immune function in older women, who were free from inflammation and had varying nutritional status with respect to nutrients of interest. This study specifically addressed the question of whether certain nutrients could predict immune response, using discriminant analysis approach. This statistical approach was advantageous in that a parsimonious predictive subset of variables can be developed, that is important in differentiating the subjects (low and high responders on various immune function variables) and at the same time allows to test the error associated in classifying individuals correctly into their groups i.e. provides misclassification rate associated with the final model. Only a few other studies have simultaneously examined and analyzed multiple nutrients with immune function in older adults, and majority of them have not found any relation between nutrients and immune function outcomes. Thus, this study contributes to the literature and shows the role of nutrition in immune function of older adults. It must be noted that due to the observational nature of this study, our results suggest only associations, not causal relationships. Experimental studies in the future could be useful to determine causal relationships between nutrients and immune function

measures. Longitudinal studies may be useful to provide the associations between nutrition and immune function over time, although these studies are time-consuming and costly. Due to blood volume considerations only few nutrients important for immune responses were included in the study. In addition, other immune function tests such as DTH response or T cell-mediated cytotoxicity, tests of health status, and dietary data could not be studied within the scope of this study. Laboratory tests of nutritional status that could serve as negative controls could not be included as most nutrients show some positive association with immune function. Further, to try to address this, we included all our nutrition variables in the final model for one variable, as an example, versus our final model. For CD4 number, by including all variables in the final model, the error rate was 28% versus the error rate of 16.4% from our final model. It is important to note that factors other than nutrition, such as exercise or stress may also influence immune function. Future studies should take these variables into account, whenever possible, for their study designs. Based on a recent study in our laboratory (Molls et al., 2003), we recognize that high intra-individual or within-person variability exists in immune function tests. In the present study, to account for the high intra-individual variability, we ensured a large sample of subjects. In addition, paired t-test for all immune function variables between day 1 and day 2 blood collection showed that immune function was not significantly different for the two days (Appendix I).

In conclusion, our study supports the notion that key nutrients such as protein, iron, zinc and vitamin B_{12} can predict immune function in older adults. Therefore, it is important that seniors maintain adequate nutritional status. This finding could be highly useful for providing messages and guidelines to older adults for maintaining health, nutritional status and quality of life.

2.5 References

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CHAPTER 3

INTER- AND INTRA-INDIVIDUAL VARIATION IN TESTS OF CELL-MEDIATED IMMUNITY IN YOUNG AND OLD WOMEN

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Inter- and intra-individual variation in tests of cell-mediated immunity in young and old women

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Abstract

Exploring means to maintain or improve immunity in older persons has been receiving attention. To establish relationships between immune function and variables of interest, it is important to determine these variables accurately and precisely. Precision relates to the degree of variation in the laboratory test. The nature and magnitude of variability in tests of immune function has not been described extensively. We examined inter- and intra-individual variation in tests of cell-mediated immunity (CMI) in generally healthy and well-nourished young (20–40 years; $n = 15$) and old (60–80 years; $n = 15$) women. Subjects provided blood samples on 2 days within a week to determine leukocyte subsets, T-cell proliferation response to phytohemagglutinin A and concanavalin A, and interleukin (IL)-1β, IL-2 and IL-6 production by stimulated mononuclear cells. Intra-individual variation was partitioned into day-to-day biological and analytical variation. Inter-individual variation was greater than intra-individual variability for most tests of CMI for both age groups. Furthermore, all CMI tests exhibited large day-to-day intra-individual variation (CV ~15% or greater) which was primarily due to biological rather than analytical sources, for both age groups. In conclusion, both age groups showed large between-person and considerable within-person variation in CMI tests. Therefore, assessment of CMI based on a single blood draw may not provide a reliable estimate of immune function.

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1. Introduction

A dysregulation in the immune system is often associated with aging. The susceptibility to many infections along with the morbidity and mortality associated with infections, increases with advancing age (Gardner and Remington, 1988; Makinodan, 1995). With an increase in the aging population, investigators are exploring means to maintain or improve immune response with aging (Buzina-Suboticanec et al., 1998; High, 1999; Lesourd and Mazari, 1999; Meydani, 1993). In order to establish the relationship of various factors with immune function, immune response tests must yield accurate and precise results. In the last two decades, significant developments have been made in establishing methodologies for accurate assessment of immune function via assays spanning acquired and innate immunity. These assays range from simple in vitro tests including quantification of lymphocytes or T-cells; T-cell proliferation response to mitogens; and cytokine production, to in vivo laboratory tests such as delayed type hypersensitivity (DTH) skin tests.

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Despite advances in the accurate assessment of various aspects of immune function, precision of these tests has not been evaluated thoroughly. Precision relates to the degree of variation in a test, which is usually described from two chief sources; inter- and intra-individual variation. Inter-individual variation, the variation that exists between different individuals, in immune function tests has been previously examined in few studies (Froebel et al., 1999; Korn et al., 1984; Malone et al., 1990; Porzsolt et al., 1983). However, there is very limited information on the intra-individual or within-person variation in commonly employed immune function tests (Fei et al., 1993; Korn et al., 1984; Malone et al., 1990; Porzsolt et al., 1983).

Variation affects assessment of immune status and statistical relationships at both the individual and the population level. At the individual level, large intra-individual or within person day-to-day variation in a laboratory test of immune function would imply that assessment based on a single day's blood collection may not provide an accurate estimate of the individual's true value for that test. At a group or population level, large variation in a laboratory test can affect statistical associations, mask differences between groups, and confound inferences. Thus, it is important to describe the extent and sources of variation in immune function tests in order to guide future investigations for precise estimation of immune function tests. Thus, the purpose of the current study was to examine the day-to-day inter- and intra-individual variability in tests of acquired immunity namely leukocyte subsets, T-cell proliferation response to mitogens, and cytokine production by stimulated mononuclear cells. Because poor health and nutritional status can affect immune response (Bogden, 1995; Chandra, 1997; Corberand et al., 1986; Lesourd, 1997; Mazari and Lesourd, 1998) we conducted this study in a cohort of generally healthy, well-nourished young and old women, who were selected for participation based on the SENIEUR protocol (Ligthart et al., 1984) as well as several nutritional status criteria.

2. Methods

2.1. Subject recruitment

Young (20–40 years; $n = 25$) and old (60–80 years; $n = 25$) women were recruited from the University and the surrounding community using flyers and advertisements in the local newspapers and television channels. Women between the ages of 41 and 59 years were not included in the study because of the potential confounding effects of hormonal changes associated with menopause on immune parameters (Hough et al., 1999; Ligthart et al., 1984; Murasko et al., 1987; Porter et al., 2001). Women receiving hormone replacement therapy were also not included in the study (Porter et al., 2001). In addition, young women who were pregnant or nursing within 6 months from the date of recruitment were not included. Subjects provided written informed consent for participation and followed protocols approved by the Office for Regulatory Compliance at the Pennsylvania State University. Subjects received an honorarium of $50 at the completion of the study.

2.2. Study protocol

Twenty-five young and 25 old volunteers were first screened for participation in the study based on a medical history to rule out any health conditions or medication use as per the SENIEUR protocol (Ligthart et al., 1984). None of the subjects were taking any dietary supplements routinely. For subjects meeting the medical history screen ($n = 18$ in each age group) fasting blood samples were collected, between 07:00 and 09:00 h, following a 15-min period of rest. Approximately 35 ml of venous blood was collected by a certified phlebotomist into three types of Vacutainer® tubes (Becton Dickinson, Franklin Lakes, NJ): tube with no anticoagulant, tube containing EDTA, and tube containing heparin. Height and weight were also recorded using portable, standardized instruments.

After screening for clinical, nutritional and inflammation status, six women (3 young and 3 old) were dropped from further participation. The reasons for excluding these subjects were: protein deficiency (2 young); vitamin B_{12} deficiency (2 old); and iron deficiency (1 young and 1 old). Therefore, the final number of subjects who were eligible to continue participation in the study was 30 (15 young and 15 old). Immune function tests were carried out for these subjects on day 1 blood collection. An additional blood sample (25 ml) was obtained from these subjects within a week of the first blood draw, to determine tests of immune function and obtain a complete blood count (CBC). Values obtained for immune function tests on the 2 nonconsecutive days were used to compute inter-individual and intra-individual variation for each age group.

2.3. Screening criteria

Eligibility for participation in the study was determined using the following screening criteria.

2.3.1. SENIEUR protocol criteria

Medical history and clinical tests of general health and inflammatory status were used to exclude individuals with acute or chronic inflammation, other medical conditions, and/or medication use known to affect immune response, in accordance with the SENIEUR protocol (Ligthart et al., 1984).

A CBC with differential evaluation on a Coulter MAZM analyzer (Beckman Coulter Corporation, Miami, FL) and clinical chemistry tests (Chem-24 profile) using a Roche Mira Plus random access chemistry analyzer (Roche Boehringer Mannheim, Indianapolis, IN) were carried out at the University Health Services Laboratory. Results of these tests were reviewed by the study physician to exclude subjects with infection, inflammation, liver disorders, kidney disorders, and/or bone marrow proliferation disorders.

To assess inflammatory status, erythrocyte sedimentation rate (ESR) was measured using the Westergren Method (Dispette72, Ulster Medical Products, Rio Rancho, NM). Serum alpha-1 acid glycoprotein (AGP) was determined by radial immunodiffusion (Kent Laboratories, Redmond, WA). Further, elevated white blood cell (WBC) count from the CBC was also considered for evaluation of inflammation. The cut-offs for defining abnormal results for these parameters were: ESR >15 mm/h for young individuals and >30 mm/h for old individuals; WBC $>11\times10^{9}$/l; and AGP >1.4 g/l (Jacobs et al., 2001). Individuals with abnormal results on two or more of these tests of inflammation were excluded from further participation.

2.3.2. Nutritional status

To assess nutritional status, laboratory tests were performed to ensure the adequacy of protein, iron, vitamin B_{12}, and folic acid. A deficiency of these nutrients is associated with impaired immune response (Chandra, 1997; Mazari and Lesourd, 1998) and therefore could affect estimates of variability. Serum total protein and serum albumin were determined as part of the clinical chemistry profile. Serum ferritin was assayed by a radioimmunoassay (Diagnostic Products, Los Angeles). Hemoglobin, hematocrit, and mean corpuscular volume (MCV) were obtained from the CBC analysis. Serum vitamin B_{12} and folate concentrations were determined by a commercial radioimmunoassay (ICN Pharmaceuticals, Orangeburg, NY).

Subjects with serum total protein <60 g/l and/or serum albumin <35 g/l were excluded from further participation (Jacobs et al., 2001). Individuals with depleted iron stores, based on serum ferritin <12 μg/l for young women and <20 μg/l for old women, and at least two other abnormal tests of iron status were considered iron-deficient and excluded from analysis (Ahluwalia et al., 1995; Milman et al., 1983). To exclude underlying vitamin B_{12} or folic acid deficiency, subjects with serum vitamin $B_{12}<184$ pmol/l, serum folate <4.5 nmol/l (Jacobs et al., 2001) and/or mean corpuscular volume >102 fl (Hillman and Finch, 1996) were dropped from analysis.

2.4. Immune function tests

For sample volume considerations, immune function tests were determined using whole blood assays previously validated in our laboratory (Krause et al., 1999).

2.4.1. Leukocyte phenotypes

Leukocyte subsets were estimated in 1 ml heparinized blood (Mandy et al., 1997) using fluorescently-labeled, monoclonal antibodies specific for surface antigens (anti-CD3 recognizes total T-cells; anti-CD4 recognizes total T-helper cells; anti-CD8 recognizes total T-cytotoxic cells; anti-CD56,16 recognizes NK cells; anti-CD19 recognizes B-cells; and anti-CD14 recognizes monocytes) by flow cytometry (Beckman Coulter EPICS XL, FL). Total lymphocyte number, obtained from the CBC with differential evaluation, was used to compute the absolute numbers of cells for various lymphocyte subsets.

2.4.2. T-cell proliferation response to stimulation with mitogens

Proliferation of T-cells in response to two concentrations each of phytohemagglutinin (PHA) and concanavalin A (Con A) were determined by measuring incorporation of [^{3}H]thymidine (6.7 Ci/mmol) (Bloemena et al., 1989; Flethcher et al., 1987). The two optimal concentrations assayed for PHA were 5 and 10 mg/l and those for Con A were 12 and 25 mg/l, respectively, based on our previous study (Krause et al., 1999). Heparinized blood was diluted (1:10) with RPMI 1640 medium containing 10% fetal bovine serum (FBS), L-glutamine (2 mmol/l), penicillin (100 000 U/L), and streptomycin (100 mg/l). One hundred μl diluted blood and 100 μl of mitogens were added to wells in a 96-well, round-bottom microtitre plate. For each mitogen at each concentration, six replicate measurements were carried out per subject on each day of blood collection. Cells were incubated for 42 h (humidified, 5% CO_2, 37 °C) followed by addition of 10 μl [^{3}H]thymidine to each well, and further incubation for another 6 h, followed by harvesting of cells onto glass fiber filters (Skatron 7025 Combi Cell Harvester, Skatron, Sterling, VA). Incorporation of [^{3}H]thymidine (CPM) into cellular DNA was determined with a Wallac 1205 beta plate counter (EG&G Wallac, Gaithersburg, MD). CPM was normalized to 1000 T-cells.

2.4.3. Cytokine determination

Heparinized blood was diluted (1:10) with RPMI 1640 medium containing 10% fetal bovine serum (FBS), L-glutamine (2 mmol/l), penicillin (100 000 U/l), and streptomycin (100 mg/l). For each subject, 2 ml of diluted blood was placed in a well and 2 ml of diluted blood and PHA (5 mg/l; PHA-M {L2646} obtained from Sigma, St. Louis, MO) was placed in another well

of a 24-well, flat-bottom microtitre plate. Cells were incubated for 48 h (humidified, 5% CO_2, 37 °C). Contents of wells were transferred into microfuge tubes, centrifuged, and supernatants collected, aliquotted, and frozen at −80 °C until subsequent assay of IL-1β, IL-2 and IL-6 as described previously (Ahluwalia et al., 2001).

For cytokine determination, samples from young and old subjects were assayed on the same plate and a pooled supernatant was used as the internal control with each run. The pooled supernatant was generated by culturing the blood of several individuals separately with PHA (as described above) and pooling the supernatants. Three replicate measurements were determined for each cytokine measurement on each day of blood collection per subject. Cytokines (IL-1β, IL-2, and IL-6) were measured by ELISA, using antibodies obtained from R&D systems. Briefly, supernatants from cell cultures with PHA were added to anti-human monoclonal IL-2 antibody coated plates (MAB602) and detected with a polyclonal goat anti-human IL-2 antibody (BAF 202). The IL-2 concentration was expressed as pg/ml of the culture supernatant. Similarly, IL-1β concentration was determined in supernatants using anti-human monoclonal IL-1β antibody coated plates (MAB601) and detected with a polyclonal goat anti-human IL-1β antibody (BAF201). The IL-1β concentration was then determined in the culture supernatant (pg/ml). For IL-6 determination, culture supernatants were added to anti-human monoclonal IL-6 antibody coated plates (R&D systems # MAB206) and detected with a polyclonal goat anti-human IL-6 antibody (R&D systems # BAF206); IL-6 concentration was expressed as pg/ml of culture supernatant. The lower limit of detection for all cytokines measured was 3 pg/ml. Cytokine levels in the supernatants were usually nondetectable in the absence of PHA for both young and old subjects. Batch-to-batch variation for the pooled supernatant was 3.6, 2.4, and 3.2% for IL-1β, IL-2, and IL-6, respectively.

2.5. Statistical analyses

Statistical analyses were carried out using the Statistical Analysis System version 8.0 (SAS Institute, Cary, NC). Most variables examined were consistent with a normal distribution with the exception of serum ferritin. Therefore, logarithmic transformed data were used for serum ferritin. Potential outliers that can exert large influence on the regression model were identified by fitting a model, examining its residuals, and assessing the leverage that each observation exerted on the fit (Gray, 1989). Proc Reg with model statement including day 1 and day 2 variables and the option 'influence' was used to identify outliers. When identified, outliers, usually no more than two observations for any immune function tests were dropped from the data set for variance component analysis.

2.5.1. Sources of variation

Variation in immune function tests was partitioned into between-person or inter-individual variability (Var_{inter}); and within-person or intra-individual variability due to: biological sources from day to day ($Var_{intraday}$); and due to analytical ($Var_{intrarep}$) sources, i.e. technical error between sample replicates, using PROC VARCOMP for young and old women separately. PROC VARCOMP is a simple SAS procedure which is used to fit separate variance component models (Searle, 1971). It computes estimates of variance components in a general linear model by using a single model statement which specifies the dependant variables and the class variables or main effects. In the current study the model statement included ID and day-nested-within ID as the main effects. The variance estimate for ID in the PROC VARCOMP output provided Var_{inter}, the variance estimate for day-nested-within ID provided $Var_{intraday}$, and the estimate for error variance provided $Var_{intrarep}$. The $Var_{totalintra}$ for lymphocyte proliferation response and cytokines production was calculated as the sum of $Var_{intraday}$ and $Var_{intrarep}$ (Ahluwalia et al., 1993).

For leukocyte subsets, analytical variation could not be calculated because replicate measurements were not carried out for each person for each day. The PROC VARCOMP for leukocyte subsets therefore included ID as the main effect which provided Var_{inter} estimate, and the error variance estimate provided $Var_{totalintra}$. The CV for each variance component was calculated by dividing the square root of the variance estimate by the mean for that particular analyte, and expressed as a percentage. The estimate of $Var_{totalintra}$ was used to calculate the $CV_{totalintra}$. Differences between young and old women on screening variables were examined using ANOVA, after controlling for appropriate covariates, namely AGP, ESR, serum ferritin and MCV.

3. Results

All subjects included in the analyses were considered generally healthy based on medical history and clinical tests of inflammation, and well-nourished based on a battery of biochemical tests for nutritional status (Table 1). Old women had higher ESR, AGP, serum ferritin and MCV than young women ($P < 0.05$); the difference observed between the two groups on these variables, however, would not be considered clinically relevant or biologically significant because the values, for both groups, fell within the normal ranges.

Immune function was assessed by quantifying the number of leukocyte subsets (Table 2), T-cell proliferation response to PHA (Table 3) and Con A (Table 4)

Table 1
Inflammation and nutritional status of study participants[a]

Index	Young ($n=15$)	Old ($n=15$)
Inflammation variables		
AGP (g/l)	0.7 ± 0.1	0.8 ± 0.1*
ESR (mm/h)	8.3 ± 6.2	15.1 ± 7.6*
WBC ($\times 10^9$/l)	6.2 ± 1.5	6.2 ± 0.9
Nutrition variables		
BMI (kg/m^2)	23.2 ± 2.8	25.1 ± 3.7
Total protein (g/l)	70.7 ± 4.0	67.8 ± 3.2
Albumin (g/l)	43.9 ± 2.5	42.7 ± 7.0
Serum ferritin (μg/l)	22.8 (13.1–39.6)[b]	60.21 (36.1–100.2)**
Hemoglobin (g/l)	136.1 ± 7.3	138.5 ± 7.8
Hematocrit (%)	40.1 ± 2.2	40.8 ± 1.9
Mean corpuscular volume (fl)	91.3 ± 2.2	94.0 ± 3.8*
Serum vitamin B_{12} (pmol/l)	425.4 ± 100.7	416.3 ± 95.4
Serum folate (nmol/l)	22.7 ± 13.4	23.4 ± 12.5

[a] Values indicate mean ± 1 S.D.
[b] Values are geometric mean; values in parentheses indicate ± 1 S.D. of geometric mean.
* Groups were significantly different: $P < 0.05$.
** Groups were significantly different: $P < 0.01$.

and the production of cytokines IL-1β, IL-2 and IL-6 by stimulated mononuclear cells (Table 5). Young and old women did not differ on any of the immune function tests ($P > 0.10$). Considerable variation was seen, however, in all immune function assays examined for both young and old women (Tables 2–5).

For various leukocyte subsets examined, inter-individual variation was greater than intra-individual variation (Table 2). The $CV_{totalintra}$ was high ($\sim 10\%$ or greater) for most leukocyte subsets for all women; however, total lymphocyte count and total T-cell numbers tended to have lower intra-individual variation ($< 10\%$). Generally, the total intra-individual variation was similar for both young and old women.

In case of proliferation response to mitogens as well, the total inter-individual variation was higher than the total intra-individual variation (Tables 3 and 4). The total intra-individual variation was rather large ($CV \sim 20\%$) and similar in both young and old subjects for lymphocyte proliferation response with PHA or Con A. The day-to-day biological variation was the major source of total day-to-day intra-individual variation compared to analytical sources. For both age groups, the analytical variation for lymphocyte proliferation was 4–5% with PHA, and 8–9% with Con A.

For all cytokines examined, IL-1β, IL-2 and IL-6 (Table 5) the variation between individuals was extremely large, especially for IL-2 production; intra-individual variation was also high ($CV \sim 15\%$ or greater) especially for IL-2, in both age groups. As for lymphocyte proliferation response, the day-to-day biological variation was the major source of total intra-individual variation relative to analytical sources. For both age groups, the analytical variation was approximately 5–8%.

4. Discussion

With an increasing older population, research efforts continue to focus on the effects of aging on immuno-

Table 2
Variation in leukocyte subsets

Leukocyte subsets	Mean ± S.D. ($\times 10^9$/l)	Var_{inter}	$Var_{intraday}$	CV_{inter} (%)	$CV_{totalintra}$ (%)
Young					
WBC	5.88 ± 1.29	1.113	0.63	17.9	13.5
Total lymphocytes	1.98 ± 0.29	0.065	0.036	12.8	9.5
Total T-cells	1.46 ± 0.56	0.307	0.017	37.8	8.9
T-helper cells	0.76 ± 0.28	0.071	0.018	35.0	17.5
T-cytotoxic cells	0.57 ± 0.18	0.033	0.006	31.8	13.9
Granulocytes	3.52 ± 0.90	0.696	0.257	23.7	14.4
Monocytes	0.36 ± 0.13	0.015	0.003	33.2	14.8
B-cells	0.13 ± 0.04	0.0014	0.0003	28.7	13.1
Natural killer cells	0.21 ± 0.07	0.0048	0.0016	31.9	18.4
Old					
WBC	6.59 ± 0.99	0.85	0.181	14.0	6.45
Total lymphocytes	2.16 ± 0.70	0.479	0.016	32.0	5.8
Total T-cells	1.35 ± 0.80	0.634	0.003	58.9	4.0
T-helper cells	0.86 ± 0.54	0.281	0.015	61.4	14.2
T-cytotoxic cells	0.54 ± 0.22	0.047	0.002	40.4	8.5
Granulocytes	3.94 ± 0.93	0.787	0.165	22.5	10.3
Monocytes	0.47 ± 0.13	0.015	0.004	25.7	13.3
B-cells	0.22 ± 0.07	0.005	0.002	32.1	21.5
Natural killer cells	0.38 ± 0.20	0.034	0.009	48.5	24.7

There were no significant differences between groups ($P > 0.10$).

Table 3
Variation in T-cell proliferation response to PHA

Index	Mean ± S.D. (cpm/1000 cells)	Var_{inter}	$Var_{intraday}$	$Var_{intrarep}$	$Var_{totalintra}$	CV_{inter} (%)	$CV_{intraday}$ (%)	$CV_{intrarep}$ (%)	$CV_{totalintra}$ (%)
Young									
PHA, 5 mg/l	53 732 ± 29 014	970 397 916	30 488 514	8 230 375	38 718 889	57.9	10.2	5.3	11.5
PHA, 10 mg/l	40 330 ± 15 906	248 031 240	60 728 849	4 038 610	64 767 459	39.0	19.3	4.9	19.9
Old									
PHA, 5 mg/l	36 251 ± 13 677	182 039 905	40 727 924	3 137 129	43 865 053	37.2	17.6	4.8	18.2
PHA, 10 mg/l	34 880 ± 20 785	383 492 388	49 657 040	2 307 166	51 964 206	56.1	20.2	4.3	20.6

There were no significant differences between groups ($P > 0.10$).

competence, and on means to maintain immune function with aging. Thus, examination of factors which can modulate immunocompetence with advancing age has been the emphasis of several immunogerontological studies. To evaluate the relationships of various factors with immune function, it is critical that immune response be quantified accurately as well as precisely. Precise estimation of an index depends upon the extent of intra-individual variation in the test (Ahluwalia et al., 1993; Beaton et al., 1989). Most observational or intervention studies usually determine immune function based on a single blood sample. A single estimate may not be sufficient to account for total intra-individual variability in the tests: in a clinical setting immune function tests carried out on a single blood sample may not provide reliable assessment of immune status for individuals; and in research settings significant associations may be missed when using tests with high variability. Thus, it is important to describe intra-individual variation in immune function tests to gain insights into the reliability of various tests; and establish whether some tests are more variable from day-to-day relative to others, to design more precise studies. This information is lacking in the literature because most previous studies have generally examined estimates of inter-individual variation only in tests of immunocompetence. Therefore, we determined inter-individual as well as intra- individual day-to-day variability in tests of acquired immunity in generally healthy, well-nourished young and old women. Immune function tests were determined in blood samples obtained on 2 nonconsecutive days over a 7-day period.

In the current study, the striking finding was that all tests of CMI examined varied considerably from day-to-day between and within subjects, regardless of age. We found that inter-individual variation was greater than intra-individual variation for all tests of CMI. This is consistent with previous findings of higher between person variation reported for NK cell cytotoxicity (Korn et al., 1984; Porzsolt et al., 1983). The comparative literature on inter-individual variability in CMI tests is limited. However, a study by Froebel et al. (1999) examined inter-individual variation in lymphocyte proliferation response to mitogens. The authors found that the magnitude of inter-individual variation in lymphocyte proliferation response to PHA was high (CV ~ 36–65%). These findings are consistent with our findings of high inter-individual variation in lymphocyte proliferation response to PHA and Con A. It is generally considered that old individuals have greater between-

Table 4
Variation in T-cell proliferation response to Con A

Index	Mean ± S.D. (cpm/1000 cells)	Var_{inter}	$Var_{intraday}$	$Var_{intrarep}$	$Var_{totalintra}$	CV_{inter} (%)	$CV_{intraday}$ (%)	$CV_{intrarep}$ (%)	$CV_{totalintra}$ (%)
Young									
Con A, 12 mg/l	11 341 ± 9572	87 122 553	5 538 682	1 156 879	6 695 561	82.3	20.7	9.4	22.8
Con A, 25 mg/l	17 121 ± 16 925	261 230 212	7 895 923	2 269 839	10 165 762	94.4	16.4	8.7	18.6
Old									
Con A, 12 mg/l	11 144 ± 7220	53 037 700	5 312 089	949 568	6 261 657	65.3	20.6	8.7	22.4
Con A, 25 mg/l	10 626 ± 6294	37 475 983	3 646 132	987 786	4 633 918	57.6	17.9	9.3	20.2

There were no significant differences between groups ($P > 0.10$).

Table 5
Variation in cytokine production by stimulated mononuclear cells

Cytokine	Mean ± S.D. (pg/ml of supernatant)	Var_{inter}	$Var_{intraday}$	$Var_{intrarep}$	$Var_{totalintra}$	CV_{inter} (%)	$CV_{intraday}$ (%)	$CV_{intrarep}$ (%)	$CV_{totalintra}$ (%)
Young									
IL-1β	221.8 ± 123.5	14 835	1379	151	1530	55.1	16.8	5.5	17.6
IL-2	154.7 ± 110.5	11 344	4476	52	4528	68.7	43.1	4.6	43.4
IL-6	2693 ± 1263	1 543 292	369 015	47 374	416 389	46.1	22.5	8.1	23.9
Old									
IL-1β	224.7 ± 96.7	8844	2161	199	2360	41.9	20.7	6.2	21.6
IL-2	272 ± 269.8	64 328	4912	350	5262	93.2	25.7	6.8	26.6
IL-6	3464 ± 1492	2 087 580	108 148	66 450	174 598	41.7	9.4	7.4	12.1

There were no significant differences between groups ($P > 0.10$).

person variation compared to younger individuals. In the current study, this trend was noted for most leukocyte subsets; interestingly, no clear effect of age on variation estimates was seen for most other immune function variables. This observation may be related to the fact that our study cohort was rather homogenous with respect to health and nutritional status.

To our knowledge, this study is the first examination of intra-individual variation in tests of CMI in both young and old women. Although few studies have addressed intra-individual variation in immune function tests (Fei et al., 1993; Korn et al., 1984; Malone et al., 1990; Porzsolt et al., 1983) the effect of age on variability, however, was not examined. In the current study, it is noteworthy that both young and old women had similar but considerable intra-individual variation (CV ~ 15% or greater) in all tests of CMI examined, namely leukocyte phenotypes, T cell proliferation response to mitogens and cytokine determination (IL-1β, IL-2 and IL-6). These results are consistent with a study by Fei et al. (1993) that examined intra-individual variation in CD4+ T helper cell numbers in HIV infected patients. Thus, our results indicate that a single estimate for immune function tests may not capture the total intra-individual variation and therefore may not provide reliable assessment.

We partitioned total intra-individual day-to-day variation into that due to biological sources and analytical error. In the current study, variation due to analytical sources tended to be small (CV ~ 4–9%) and similar to that reported by Froebel et al. (1999). Although there is a lack of comparative literature on biological and analytical sources of intra-individual variation in immune function tests, our observation of biological sources being the chief source of day-to-day intra-individual variation is consistent with the findings of previous reports examining variability in iron status indices (Ahluwalia et al., 1993; Borel et al., 1991).

An interesting point to consider is whether inter- and intra-individual variation estimates for lymphocyte subsets are different when they are expressed as percentages, instead of absolute cell numbers. It may be speculated that the variation (inter- and intra-individual) is lower in lymphocyte subsets when expressed as percentages than in lymphocyte subsets expressed as cell numbers, as the latter is computed as percent subset multiplied by lymphocyte number. We found that indeed the variation estimates in lymphocyte subsets expressed as percentages were generally lower (data not shown). However, a high inter-individual variation in lymphocyte subsets (%) was seen in both young and old women (CV_{inter} ranged from 17 to 52%) and the trend of high intra-individual variation also persisted ($CV_{totalintra}$ ranged from 3 to 14%). Thus, the study findings of high inter- and intra-individual variation remained the same even when lymphocyte subsets were expressed as percentages.

Variability in tests of CMI such as leukocyte subsets and T-cell function can be influenced by several factors. These include subjects' health and nutritional status, other environmental factors such as smoking, stress, exercise, and physiological factors including diurnal variation (Bertouch et al., 1983; Gupta and Good, 1986; Landman et al., 1984; LaPierre et al., 1991; Meuleman and Katz, 1985; Saunders, 1985; Schindler, 1985; Signore et al., 1985). The current study was designed to control for the effects of health, nutrition, smoking, and biological factors related to estrogen levels and diurnal variations in study participants. Thus, this study does not address the specific effects of poor health, single or multiple nutrient deficiencies, stress, exercise and other biological factors on variation in immune function tests. Future studies are needed to examine the effect of these factors on variability in immune function tests. The findings from the current study are therefore applicable to generally healthy, well-nourished women. We recognize that the estimates of inter- and intra-individual variation for both age groups from this study may be conservative for use with a more heterogenous group.

The current study suggests that although some biological variation in tests of immune function is expected, most commonly employed tests of CMI show considerable day-to-day intra-individual variation for both young and old, generally healthy and well-nourished, women. Thus, a single estimate of immune function may not yield precise assessment. The effects of high intra-individual variability can be attenuated, at the individual and population level, by increasing the number of days of blood sampling (Liu and Liang, 1992). Alternatively, the attenuating effects of high intra-individual variability in immune status indicators in surveys and studies can be compensated by ensuring adequate sample size to account for expected variation between individuals and variation within individuals due to biological and analytical sources (Beaton et al., 1989). In conclusion, this study provides estimates of both inter- and intra-individual variation in commonly employed tests of CMI, which could assist in determining sample size and number of replicates for various tests in future studies examining immune function.

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CHAPTER 4

FUTURE DIRECTIONS

This thesis is comprised of two studies described in Chapters 2 and 3. The focus of the first study was to examine the relationship between nutrients important for immune function, protein, iron, zinc, vitamin B_{12} and folate, and tests of acquired immunity, such as T cells and subsets, lymphocyte proliferation response, and cytokine production. The specific objective of the study was to obtain a subset of nutrients, which can classify healthy older women as low or high responders on the tests of acquired immune function, by using discriminant analysis approach. This statistical approach of discriminant analysis showed that nutrients such as protein, iron, zinc, folate and vitamin B_{12} are important for certain immune functions of older adults in our study.

This study, although carefully controlled to determine the relationship between nutrition and immune function in older women, presented few limitations and need for future investigations. Future studies could be aimed at the following:

- Including dietary and biochemical data of multiple nutrients important for immune function; evaluation of innate and acquired immune function by other tests such as chemotaxis, phagocytosis and bactericidal function, NK and T cell cytotoxicity, DTH responses; and examination of clinical endpoints such as infection.
- Controlling for factors other than nutrition such as exercise or stress to study relationship between nutrients and immune function
- Designing experimental studies i.e. experimental manipulation of nutrient levels such as a repletion-depletion, to determine causal relations between various nutrients measured simultaneously and immunity.
- Investigating longitudinal data on nutrition and immune function outcomes in older adults, as such data is extremely limited

- Determining mechanisms by which multiple nutrients, and interactions between them, predict or influence immune function outcomes.

The focus of the second study of this thesis was to examine the day-to-day inter- and intra-individual variability in tests of acquired immunity namely leukocyte subsets, T-cell proliferation response to mitogens, and cytokine production by stimulated mononuclear cells, in a cohort of generally healthy, well-nourished young and old women. The findings from this study showed that in both age groups all cell-mediated immune tests exhibited large inter-person and considerable day-to-day intra-individual variation. Future studies could include other factors that could influence variability in immune function tests such as poor health and infections, gender, nutrient deficiencies, stress etc. In addition, these studies should determine intra-individual variation in immune function indices with at least two or more blood collections, and further, using replicate measurements for each blood sample to determine the analytical sources of variation in all immune function tests included. This study on variability was conducted prior to the study in Chapter 2. Based on findings from this variability study, for the subsequent study on discriminant analysis (Chapter 2), to account for the high intra-individual variability in immune function, we ensured a large sample of subject, in addition to controlling factors that could affect variability in immune function such as health and stress. In addition, paired t-test for all immune function variables between day 1 and day 2 blood collection in Chapter 2 showed that immune function was not significantly different for the two days (Appendix I). Therefore, findings from our study on variation in immune function (Chapter 3) were applied to study design of Chapter 2.

APPENDIX A
OVERALL FLOWCHART FOR SUBJECT RECRUITMENT FOR CHAPTER 2

	Study 1	Study 2	
1. Subjects who received invitation letters or phone calls to participate	~ 300	~ 70	~ 400
2. Subjects who were interested in participation or replied back to letters	85	30	120
3. Subjects on whom medical histories were obtained	75	25	80
4. Subjects eligible to participate after medical history screening	71	20	65
5. Subjects who provided blood*	71 (59 HBO and 12 FLO)	18 FLO	48 (44 HBO and 4 FLO)
6. Subjects who had no inflammation	69 (57 HBO and 12 FLO - 2 HBO had high AGP and WBC and were dropped)	18 FLO	43 (39 HBO and 4 FLO - 5 HBO with high ESR were dropped before immune tests)
7. Subjects who completed the study	69 (57 HBO and 12 FLO)	18 FLO	43 (39 HBO and 4 FLO)
8. Total number of subjects in each study	69	18	43

*HBO – homebound older women, FLO – free-living older women
Total N = 69 + 18 + 43 = 130

APPENDIX B
MEDICAL HISTORY FORM

Study ID #: ____________SS# : ____________________________ Date: ____________

Name: ____ Age: _________________

Home Address: | **Family Doctor's Address:**

_______________________________________ _____________________________________

_______________________________________ _____________________________________

_______________________________________ _____________________________________

_______________________________________ _____________________________________

Home Telephone: | **Family Doctor's Telephone:**

_______________________________________ _____________________________________

Medical History

NOTE: Acceptance or decline of the subject to participate in the study will not be confirmed during the medical history, but followed up with a later phone call and/or letter. Interviewer to make a note of all conditions, regardless of whether they are likely to affect the subject's eligibility to participate. This form will be reviewed by the study physician to determine further eligibility to participate in the study.

Have potential subjects been diagnosed with these conditions (if so when, and do they currently have that condition):

1. Lungs / breathing problems – active tuberculosis, asthma, pneumonia, active emphysema / COPD / other lung conditions or on oxygen, current or recent bronchitis, allergies, orthopnea, dyspnea

2. Heart condition (describe) – angina, irregular heart rate, CHF, high blood pressure, heart attack

3. Circulation: Leg ulcers, edema (swelling), high blood pressure, varicosities, peripheral vascular disease, and cerebral insufficiency

4. Lymph nodes – enlargement, HIV positive, SLE (Lupus)

5. Inflammatory bowel disease (ulcerative colitis, Crohn's disease, etc.), gastrointestinal conditions (peptic ulcers, diverticulosis, etc.) colitis, intestinal problems, jaundice, gallbladder disease, (GERD) / hernia

6. Anorectal disorders – hemorrhoids, prolapse, fistulas, fissures, etc.

7. Musculoskeletal – fractures, osteoporosis, osteomyelitis, osteoarthritis, rheumatoid arthritis (distinguished from osteoarthritis by symmetry on both sides of body), bone infection or recent infections involving the bone

8. Skin infections: bruises, rashes, skin allergies

9. Nervous system: stroke, Parkinson's or Alzheimer's disease, multiple sclerosis, polio history, seizeurs, depressions

10. Endocrine - diabetes (note child or adult onset, whether taking insulin, etc), disease of thyroid, spleen, or pancreas, metabolic disorders, liver disease / jaundice (total bilirubin > 3 or biopsy – proven liver disorder)

11. Kidney disease (creatinine > 2 or on dialysis), current or recent (within the past 3-4 weeks) urinary track infection, kidney failure / infection

12. Cancer (breast, colon, etc.) cervical, gynecological, leukemia, tumors, lymphoma, Hodgkin's disease

13. Gout

14. Vitamin B_{12} or folic acid deficiency

15. Anemia (If so, please describe, e.g., due to iron deficiency or other causes or any other blood disease?)

16. Any other not mentioned, say communicable diseases. Due to what?

Other questions:

1. Have you has a previous history of anemia or poor blood or low iron in blood?

2. Do you have a currently have anemia or poor blood or low iron in blood?

3. Have you previously or currently been prescribed any iron supplements?

4. Are you vegetarian?

5. Do you eat red meats? If yes, how often?

6. Menstrual history: Have you had a history of heavy blood loss during menstruation?

7. Are you taking any antibiotics currently or within the past month? For what conditions? When did you stop taking them?

8. Have you had a history of any of the following?

- Organ transplants (ever)
- Blood transfusion in the previous 3 months

9. Do you currently have any other infections or fever (cold / flu / etc.), which were not included in the above questions? If so, describe.

10. Are there any other conditions you have which you see a doctor for periodically?
Interviewer: Please make a note of all conditions, regardless of whether they are likely to affect the subject's eligibility to participate.

List all medications that the subject is currently taking, or has taken in the past several weeks, in the space below. Indicate vitamin/mineral supplements. Include descriptions (for what condition is the medication taken, how often, etc.).

Currently **Within past month**

APPENDIX C
INFORMED CONSENT FOR CLINICAL RESEARCH STUDY

The Pennsylvania State University

Title of Project: Iron Status and Immune Response in Older Women

Principal Investigator: Naman Ahluwalia, Ph.D.
Nutrition Department
Phone: 863-5830

Other Investigators: Gordon Handte, M.D.
Director, Clinical Laboratory,
University Health Services

This is to certify that I, ______________________________, have been given the following information with respect to my participation as a volunteer in a program of investigation under the supervision of Drs. Naman Ahluwalia and Gordon Handte.

1. Purpose of the study:

The purpose of this study is to examine the relationship between iron status and immune function in older women. The study will also determine whether taking iron supplements will also improve immunity or resistance to fight infections in iron-deficient women. About 200 women (60-85 y) will be recruited into this study.

2. Procedures to be followed:

My participation in this study (Study 1) will involve the following:

A) Giving a medical history including what kind of medicines and/or nutrient supplements I take, and going over a list of conditions over the telephone or in person to the research assistant to make sure that I do not have any of the conditions which can interfere with the blood tests;

B) Visit 1: Screening. A technician trained to draw blood from my finger will visit me at my home on the scheduled day of our appointment. I will provide a small blood sample from my finger, which will be used to determine the hemoglobin/ iron level in my blood on a portable machine at my home. The results of this test will be used to determine whether I may be eligible to participate in the study.

C) If the prescreening finger prick hemoglobin test is low (< 11.5 g/dL) suggesting that I may be iron deficient, I will be invited to participate in this project. If the finger prick hemoglobin test result is normal I may be contacted at a later time to still participate in the program depending on the number of participants still needed for the project.

D) Visit 2: If I am invited and accept to continue in the program, a graduate student and a person trained to draw blood (technician) will visit me at my home on the scheduled day of our appointment. For this visit, I will:

i) Not eat any food or beverage (except water) from midnight to the time a blood sample (35 ml or about 7 teaspoons) is taken from my arm in the morning by the technician at my home which will be sent to laboratories at the Pennsylvania State University for tests of iron status and my body's ability to fight infections, <u>at no cost to me</u>; and

ii) Keep a record of all I eat and drink (except water) on the day before the scheduled visit. I will give this food record to the graduate student and she will ask me further questions regarding the foods I ate on the previous day. If I am not able to write my food record, my family or friend may write this information for me. Otherwise, the graduate student will simply ask me questions verbally about what I ate on the day before the visit to my home. If there are no physical problems with me providing my height and weight, the graduate student will take these measurements, using a portable weighing scale and a tape measure, and record them for the study.

I give my permission to forward a copy of my laboratory test results to my family doctor. I will provide the name and address of my family doctor or primary health care provider for this purpose.

E) I will be contacted by the graduate student by telephone 2-3 days following the blood collection and told whether I can participate further in the study based on the results of my blood tests. If any of the test results are found to be abnormal, a letter will be sent by the physician involved in this study to my family doctor so that I can receive further medical care, if needed.

F) Visit 3: If my test results verify that I am eligible for further participation, a visit will be scheduled within 5-7 days of visit 1. At this visit the graduate student and a person trained to draw blood (technician) will visit me again at my home. For this visit, I will:

i) Not eat any food or beverage (except water) from midnight to the time a blood sample (15 ml or about 3 teaspoons) is taken from my arm in the morning by the technician at my home which will be sent to laboratories at the Pennsylvania State University for tests to determine my body's ability to fight infections, at no cost to me; and

ii) Keep records of all I eat and drink (except water) for the two days before the scheduled visit. I will give these food records to the graduate student and she will ask me further questions regarding the foods I ate on the previous two days. If I am not able to write my food records, my family or friend may write this information for me. Otherwise, the graduate student will simply ask me questions verbally about what I ate on the two days before the visit to my home.

Based on the laboratory work done in this project (study 1), if my blood tests show that I am iron-deficient and anemic (hemoglobin < 11.5 g/dL), I will be eligible to participate in a follow-up program (Study 2). Based on my hemoglobin levels I will qualify to do either option 1 or 2 of this program.

Iron deficiency can be due to 1) inadequate intake of iron or other nutrients 2) blood loss due to medical conditions, including cancer of the gastrointestinal tract (i.e. colon cancer) or 3) related to medication use. If I am found to be iron-deficient, the study physician (Dr. Handte) will notify my family doctor, by registered mail, to provide me necessary follow-up to determine the cause of my iron deficiency and to rule out the possibility of cancer.

Dr. Handte will also call my family doctor by phone, to indicate that I am participating in the iron supplementation program and to check that my doctor will do a follow-up to determine the cause of my anemia. If I chose to participate in either Option 1 or 2 involving taking iron supplements (described in detail below), taking iron tablets for 2 months may correct the iron deficiency anemia but will not treat any other disease(s) that may be causing the anemia. Therefore, it is very important that I contact my family doctor for a detailed work up to rule out other potentially more serious causes of iron deficiency such as cancer, since taking iron tablets may correct my anemia but it will not treat the disease.

Study 2 -Option 1: If my blood tests show that I am iron-deficient and mildly anemic (hemoglobin between 9 to 11.5 g/dL), and decide to continue further in the supplementation project (Study 2), my participation will involve the following:

A) At visit 3, I will be given iron supplements, at no cost to me, by the graduate student along with typed instructions on how much and when to take it. I will take one tablet every day before going to bed at night or 3-4 hours after my main evening meal (supper) so that I get the maximum benefit from the supplement.

B) While I am taking the supplement tablets, there is a small chance that I may initially feel heaviness/ bloating in the stomach, or get diarrhea or constipation. If I experience any of these problems while my body is adjusting to taking the supplement, I will contact the graduate student or Dr. Ahluwalia at 863-5830 to let them know about any problems that I may be experiencing. They will immediately call me back and advise me on the phone about how to overcome these problems, e.g. taking the tablet once every other day for a few days up to a week after starting taking the supplement and/or taking it with my dinner. Once these problems subside, usually in a few days to a week, I will take the tablet (supplement) every day as described before.

C) Within a week of my starting the iron supplement program, the graduate student will call me and ask me about how I am feeling and also make another appointment to visit me in another week to 10 days. Depending on how I feel and my symptoms, the dose of iron supplement and time of taking the supplement will be adjusted to avoid any discomfort.

D) Visit 4: A graduate student will visit me at my home on the scheduled day of our appointment. For this visit, I will:

i) Keep a record of all I eat and drink (except water) for the previous day before the scheduled visit. I will give this food record to the graduate student and she will ask me further questions regarding the foods I ate on the previous day. If I am not able to write my food record, my family or friend may write this information for me. Otherwise, the graduate student will simply ask me questions verbally about what I ate on the day before the visit to my home.

ii) Be given iron supplements by the graduate student for the next 10-15 days.

E) The graduate student will also answer any questions that I may have, and collect any leftover iron tablets from the previous supply.

F) The graduate student will mail supplements for another 2 weeks to me before visit 5. The graduate student will call me during this time to make sure that I received my supplements, and check with me on how I feel and that I am taking my supplement. She will also schedule the next appointment (Visit 5) at my home.

G) Visit 5: This visit will involve the same things as visit 3 (supplements provided and dietary record noted). The last batch of supplements will be provided to me at this time (for 15 days). I will also schedule the next visit with the graduate student.

Post-intervention

H) Visit 6. This visit will involve the same things as visit 2 i.e., a dietary record will be obtained and a single blood draw of 30 ml or 6 teaspoons will be taken by the trained technician for tests of

iron status and my body's ability to fight infections, at no cost to me. A final visit will be scheduled within 6 days after visit 6.
I) Visit 7. This visit will involve the same things as visit 3, i.e. a single blood draw of 15 ml or 3 teaspoons will be collected by the trained technician for tests of my body's ability to fight infections, at no cost to me.

J) The test results from visits 6 and 7 will be reviewed by the study physician, Dr. Handte. Copies of the test results will be mailed to me and my physician. If any of the test results are abnormal my physician will be notified for further follow-up.

Study 2 - Option 2: If my blood tests show that I am iron-deficient and severely anemic (Hemoglobin < 9 g/dL), a letter will be sent along with my test results to my family physician by the study physician, Dr. Handte, recommending follow-up and suggesting need for iron supplementation and usual care, to be provided by the family physician.

If I decide to continue further in this project, my participation will involve the following:

A) The graduate student will contact me by phone after I finish study 1 to find out if my physician has prescribed me iron supplements. If I decide to take iron supplements as recommended by my physician, I will inform the graduate student the type and dose of supplements that I am taking.

B) I will schedule four additional visits (visit 4 to visit 7) with the graduate student to monitor my dietary intake, and for blood collection for tests of iron status and immune function as described below.

C) Visit 4: A graduate student will visit me at my home on the scheduled day of our appointment about 1 to 2 weeks after I start taking iron supplements. For this visit, I will:

i) Keep a record of all I eat and drink (except water) for the previous day before the scheduled visit. I will give this food record to the graduate student and she will ask me further questions regarding the foods I ate on the previous day. If I am not able to write my food record, my family or friend may write this information for me. Otherwise, the graduate student will simply ask me questions verbally about what I ate on the day before the visit to my home.

ii) The graduate student will also answer any questions that I may have, and ask me questions regarding how I feel, how many iron tablets I take every day, how often I take the tablets, and whether I take them with or without food.

D) Another visit will be scheduled in 3-4 weeks when the graduate student will call me (Visit 5). I will keep a food record and answer the graduate student's questions regarding my supplement use as in visit 4. During this visit, I will also schedule the next visit with the graduate student.

E) Visit 6. This visit will involve the same things as 2 i.e., a dietary record will be obtained and a single blood draw of 30 ml or 6 teaspoons will be taken by the trained technician for tests of iron status and my body's ability to fight infections, at no cost to me. A final visit will be scheduled within 6 days after visit 6.
F) Visit 7. This visit will involve the same things as visit 3, i.e. a single blood draw of 15 ml or 3 teaspoons will be collected by the trained technician for tests of my body's ability to fight infections, at no cost to me.

G) The test results from visits 6 and 7 will be reviewed by the study physician, Dr. Handte. Copies of the test results will be mailed to my physician and me. If any of the test results are abnormal my physician will be notified for further follow-up.

3. Discomforts and risks:

I may experience slight discomfort from the finger prick blood collection. If I provide a blood sample from my arm, I may also experience slight discomfort from the insertion of needle into my veins for collecting blood sample. Temporary bruising may occur and there may be a small possibility of infection. Standard sterile techniques will be used to prevent this from happening. The technician in the study has extensive experience in collecting blood samples safely with minimum discomfort.

I may also experience some discomfort in the stomach, temporary feeling of heaviness in the stomach, diarrhea, or constipation, while taking iron supplements. However, these symptoms usually go away with time and may not occur with the amount of supplement given in the study. The stools may appear dark brown or black while I am taking the supplement but that is not known to have any adverse effects.

If I finish study 2, it is also likely that once I stop taking iron tablets, my anemia may return. Therefore, I will keep in touch with my family doctor for regular check ups and necessary follow-up even after the completion of this project.

4a. Benefits to me:

I will benefit from participation in a scientific study and gain knowledge about my iron and vitamin (B_{12} and folic acid) status. These nutrients are important in maintaining my health and preventing anemia. I will also learn about my kidney and liver function and my dietary intake. All blood tests will be done at no cost to me or my insurance and the supplement will also be provided to me free of cost.

4b. Potential benefits to society:

Iron deficiency remains a nutritional problem particularly among the vulnerable groups such as homebound older women. This study will examine the relationship of iron status and body's ability to fight disease among older women who are receiving assistance from the Area Agency on Aging. Identification of iron deficiency is important because one can improve dietary iron intake, and/or take iron supplements to prevent the development of anemia. Iron deficiency and anemia are associated with reduced capacity to work, tiredness, and lack of energy.

Society will benefit because this study will provide important information on the relationship between dietary factors and iron status, and between iron status and immunity among older women. Further whether iron-deficient older women can benefit from iron supplements in improving their body's ability to fight infections will also be established.

5. Alternative procedures which could be utilized:

There are none; you can choose to not participate.

6. Time duration of the procedures and study:

It will take about 1 hour at the initial interview and verbal explanation of the study. There will be three visits to see me at my home, each visit will last about 1 hour. It may take me about an hour to keep a food record (or a verbal food recall for me if I have physical difficulty in writing the record). In all, three food records will be collected requiring 3 hours of my time over the course of the study (Study 1). Thus, the total time I may spend on this study (Study 1) will be about 7 hours.

If I am eligible to participate in study 2, four additional visits will be scheduled. Each visit will last about an hour (total of 4 hours). I will be asked to provide three additional food

records requiring about 3 hours of my time. Another 8 hours may be required over the 2 month period in which I will be contacted over the phone to ask me about how I am feeling, and the time required me to take my supplement every day. Thus, an additional 15 hours will be required for the supplementation part of this project.

7. Statement of Confidentiality:

My participation in this research is confidential. Only the investigator, the study physician (Dr. Handte), the graduate student, and the research assistant will have access to my identity and to any information associated to me. All records associated with my participation in the study will be subject to the usual confidentiality standards applicable to medical records, and in the event of any publication resulting from this research no personally identifiable information will be disclosed.

8. Right to ask questions:

I have been given the opportunity to ask any questions that I may have, and all such questions or inquiries have been answered to my satisfaction. If I have any further questions or concerns during the study I can contact Dr. Ahluwalia at 863-5830 or the graduate student at 865-2786.

9. Compensation:

If I finish study 1, I will receive up to $100 depending on my participation ($10 for providing the finger prick blood sample; $50 for providing first blood sample from my arm, screening information and 24-hr diet record; and additional $40 for providing the second blood sample and two more 24-hr diet records). If I am also involved in the supplementation trial (Study 2), I will receive up to an additional $150, depending on the extent and duration of my participation in the program. For the 8 weeks of taking daily iron supplements I will receive $50; and for providing 2 blood samples and 2 days of dietary information at the end of the 2 months supplementation program, I will receive an additional $100 at the completion of the study. If I stop participation in the study any sooner for personal reasons or in the event of a health event (hospitalization, injury, or falling sick) I will receive an honorarium adjusted for the duration of my participation in the study.

If I am an employee of Penn State University, the compensation that I will receive for participation will be treated as taxable income and therefore taxes will be taken from the total amount. If I am not employed by Penn State University, total payments within one calendar year that exceed $600 will require the University to annually report these payments to the IRS. This may require me to claim the compensation that I will receive for participation in this study as taxable income.

10. Injury statement:

If I stop participation in the study any sooner for personal reasons or in the event of a health event (hospitalization, injury, or falling sick) I will receive an honorarium adjusted for the duration of my participation in the study. I understand that medical care is available in the event of injury resulting from research but that neither financial compensation nor free medical treatment is provided. I also understand that I am not waiving any rights that I may have against the University for injury resulting from negligence of the University or investigators. Questions regarding this statement or my rights as a subject of this research should be directed to the Office for Research Protections in 212 Kern Building, University Park, PA (814-865-1775).

11. Voluntary participation:

I understand that my participation in this study is voluntary, and that I may withdraw from this study at any time by notifying the investigator. I also understand that I may decline to answer specific questions or decline to perform certain tasks. I also understand that my

participation in this research may be terminated by the investigators without regard to my consent if I am unable or unwilling to comply with the guidelines and procedures explained to me. During the study period if any health event such as hospitalization, injury or other illness occurs I may be asked to withdraw from the study.

This is to certify that I consent to and give permission for my participation as a volunteer in this program of investigation. I understand that I will receive a signed copy of this consent form. I have read this form, and I understand the content of this consent form.

____

Volunteer's or Proxy's* signature Date

* Proxy (in case you can not sign yourself, a person assigned proxy by you can sign for you on this line).

I the undersigned have defined and explained the study to the above volunteer.

____

Investigator's signature Date Witness's signature Date

APPENDIX D
OVERALL FLOWCHART OF IDS FOR SUBJECT RECRUITMENT

Subjects who provided blood
(n = 137)

Subjects who were dropped due to inflammation*
(n = 7)
IDs: 1, 76, 503, 507, 510, 511, 520

Final study cohort**
(n = 130)
IDs: 2, 3, 4, 8, 14, 20, 21, 22, 31, 32, 33, 34, 37, 38, 42, 43, 44, 45, 46, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 64, 65, 66, 67, 68, 69, 70, 71, 72, 75, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 501, 502, 504, 505, 506, 508, 509, 512, 513, 514, 515, 516, 517, 518, 524, 525, 526, 527, 528, 532, 533, 534, 535, 539, 542, 545, 557, 559, 560, 561, 562, 563, 564, 565, 566, 567, 568, 569, 570, 571, 572, 573, 574, 712, 713, 714, 724, 725, 726, 727, 728, 729, 730, 731, 732, 733, 735, 736, 737, 740, 743

*Inflammation was considered present if 2 of 3 tests were positive: ESR > 30 mm/h; WBC > 11 x 10^9/L; and AGP > 1.4 g/L
**Data on IDs numbered 501 through 574 were collected by the author and Amy Miltko

APPENDIX E
IDS CONSIDERED HAVING LOW PROTEIN STATUS[A,B]

ID number (n = 28)	Body mass index (kg/m^2)	Serum protein (g/L)	Serum albumin (g/L)
8	**16.5**	71	48
32	**16.4**	80	46
68	**17.8**	65	42
69	31.2	**59**	37
82	24.3	**59**	35
87	23.5	**57**	35
88	23.9	**54**	35
92	**15.5**	74	41
94	**17.8**	71	43
102	**16.3**	72	40
106	20.7	**58**	39
505	**19.5**	72	47
557	**14.8**	70	45
559	**18.5**	63	40
562	**16.4**	73	46
568	**19.9**	71	44
569	**19.5**	70	46
570	**18.4**	68	42
571	**18**	67	40
572	**19.4**	73	41
574	**19.6**	71	42
724	**18.0**	68	37
725	27.9	65	**33**
729	**18.2**	67	40
730	20.4	66	**34**
732	**19.2**	65	40
733	24.6	**57**	35
740	23.4	**57**	36

[a]Low protein status is based on body mass index (BMI) < 20 and/or serum protein < 60 g/L and/or serum albumin < 35 g/L (Ligthart et al., 1984; Jacobs et al., 2001)
[b]Values below the deficiency cutoff for each test are bolded

APPENDIX F
IDS CONSIDERED HAVING LOW VITAMIN B_{12} AND FOLATE STATUS[a,b]

ID number (n = 12)	Serum vitamin B_{12} (ng/L)	Serum folate (μg/L)	MCV (fL)
20	.	.	**104**
65	649.5	17.2	**103**
66	314.7	4.0	**103**
88	**242.7**	23.8	91
504	**99.0**	17.8	88
508	**164.3**	15.1	85
515	**240.2**	15.2	88
559	**109.7**	21.6	88
564	**168.1**	5.1	87.5
569	869.8	14.5	**103.5**
572	**93.8**	14.4	92
731	260.8	**1.9**	86

[a]Low vitamin B_{12} and folate status is based on serum vitamin B_{12} < 250 ng/L and/or serum folate < 2 μg/L and/or MCV > 102 fL (Carmel, 1988; Hillman and Finch, 1996; Jacobs et al., 2001)
[b]Values below the deficiency cutoff for each test are bolded

APPENDIX G
IDS CONSIDERED HAVING LOW ZINC STATUS[a,b]

ID number (n = 7)	Plasma zinc (µg/L)
79	60.68
88	**67.49**
100	**70.22**
712	**70**
724	**70**
735	**64**
740	**64**

[a]Low zinc status is based on plasma zinc < 700 µg/L (Gibson, 1990)
[b]Values below the deficiency cutoff for each test are bolded

APPENDIX H
IDS CONSIDERED HAVING LOW IRON STATUS[a,b]

ID	Serum Ferritin µg/L	Serum TfR mg/L	Serum Iron µg/L	TIBC µg/L	TS %	RBC k/uL	Hb g/L	Hct %	MCV fL	MCH pg	MCHC g/dL	RDW units
	< 20	> 8.5	< 50	> 350	< 16	< 4.2	< 120	< 36	< 80	< 28	< 32	> 14
Iron depletion (n = 5)												
106	**11.6**	4.87	63	316	20	4.94	144	43.7	88	29	33	13.9
504	**16.77**	4.98	113	349	32	5	139.1	41.72	88	29	33	13.8
539	**19.47**	7.53	106	332	32	6.01	143.7	43.12	91	30	33	11.9
559	**19.17**	7.74	69	236	23	4.77	125.2	37.57	88	29	33	13.2
569	**11.36**	3.07	127	159	44	4.2	134.5	40.34	103.5	34.5	33.5	11.3
Tissue iron deficiency (n = 4)												
506	32.86	**8.62**	107	303	35	4.81	140.6	42.19	87	29	33	12.4
512	116	**10.81**	69	337	20	4.96	136.7	41.01	88	30	34.5	12.35
560	86.03	**8.58**	66	222	23	4.2	124.2	37.25	98	33	34	13.1
572	**19.29**	**8.96**	84	213	28	4.43	124.9	37.47	92	30.5	32.5	13.35
Iron deficient erythropoiesis (n = 12)												
33	**15**	**10.59**	**43**	**383**	**11**	5.06	147	45	89	29	33	**15.2**
37	**18.85**	5.95	**42**	302	**14**	4.46	135	41.1	92	30	33	12.4
38	**15**	4.36	**17**	277	**6**	4.68	147	43.9	94	31	34	11.9
79	**14**	**14.77**	59	**364**	16	4.98	139	44.2	89	28	**31**	13.6
83	**9.3**	8.22	75	**393**	19	4.47	142	43	96	32	33	12.7
84	**16**	6.08	**42**	293	**14**	4.42	127	38.9	88	29	33	13.8
514	141.1	**9.64**	70	**359**	19	4.37	133.7	40.1	94	35.5	37.5	13.75
545	**0.75**	5.41	**34**	**371**	**8**	4.3	125.8	37.73	80.5	**26.5**	33	**14.8**
557	**3.24**	**9**	**37**	277	**12**	4.26	127.6	38.27	91.5	29.5	32.5	12.1
526	123.4	**9.95**	77	**359**	21	**3.98**	140.1	42.03	92.5	31	34	12.15
567	26.39	**9.1**	**41**	**361**	**10**	**3.44**	128.8	38.65	91	30	33	13.9
573	29.92	**8.83**	**40**	**357**	**10**	4.48	134.9	40.47	89.5	29.5	33	12
Iron deficiency anemia (n = 12)												
52	**8.5**	7.95	**49**	293	17	**3.5**	**116**	**34.1**	98	33	34	13.4
61	**10.7**	7.88	**42**	250	**12**	**3.77**	**105**	**33.2**	88	28	32	**16**
88	**18.6**	**9.96**	**35**	252	**14**	**2.91**	**90**	**26.4**	91	31	34	**14.2**
91	**11.4**	7	**36**	257	**14**	4.25	**119**	**35.2**	83	28	34	**14.2**
99	**11.1**	**9.51**	**44**	**358**	**12**	4.38	**117**	**35.8**	82	**27**	33	**14.8**
103	**10.2**	6.72	60	313	19	**3.94**	**118**	**35.2**	89	30	34	13.5
562	**5.37**	**14.08**	**26**	287	**8**	4.35	**118.7**	**35.62**	87	29	33	**14.4**
563	**0.34**	4.82	**28**	272	**9**	**3.82**	**119.3**	**35.78**	93	30.5	33	13.95
731	**4.4**	**11.13**	**30**	**409**	**7**	4.35	**119**	**35.6**	86	29	33	**15.2**
561	29.87	**10.95**	**41**	292	**12**	4.83	**115.6**	**34.69**	80.5	**26**	32.5	**16.05**
565	371.47	**14.62**	62	159	28	**3.77**	**113.9**	**34.17**	94	30	32	**15**
566	186	**10.04**	**34**	214	**14**	4.65	**114.4**	**34.33**	86	28	32	**14.4**

[a]Low iron status is based on 4 criteria (Ahluwalia et al., 1995; Jacobs et al., 2001; Milman et al., 1983):
Iron depletion: serum ferritin < 20 µg/L
Tissue iron deficiency: serum ferritin < 20 µg/L and serum transferrin receptor (TfR) > 8.5 mg/L
Iron deficient erythropoiesis:
serum ferritin < 20 µg/L and abnormal transport iron parameters (serum iron < 500 µg/L, TIBC > 3500 µg/L, and TS < 16 %) OR
serum TfR > 8.5 mg/L and abnormal transport iron parameters (serum iron < 500 µg/L, TIBC > 3500 µg/L, and TS < 16 %)
Iron deficiency anemia:
serum ferritin < 20 µg/L, abnormal transport iron parameters (serum iron < 500 µg/L, TIBC > 3500 µg/L, and TS < 16 %) and red blood cell indices (hemoglobin < 120 g/L, or hematocrit < 36 % or mean cell volume < 80 fL) OR
serum TfR > 8.5 mg/L, abnormal transport iron parameters (serum iron < 500 µg/L, TIBC > 3500 µg/L, and TS < 16 %) and red blood cell indices (hemoglobin < 120 g/L, or hematocrit < 36 % or mean cell volume < 80 fL)
[b]Bolded values are indicative of low iron status using that particular test

APPENDIX I
PAIRED T TEST RESULTS FOR LEUKOCYTE AND SUBSETS, LYMPHOCYTE PROLIFERATION RESPONSE TO PHA AND CON A, AND CYTOKINE PRODUCTION BY STIMULATED MONONUCLEAR CELLS

1. Leukocyte and subsets

Difference in value (day 2 – day 1)	N	P-value
WBC (x 10^9/l)	46	NS
Lymphocytes (x 10^9/l)	46	NS
Granulocytes (x 10^9/l)	46	NS
Monocytes (x 10^9/l)	46	NS
Total T-cells (%)	46	NS
Total T-cells (x 10^9/l)	46	NS
T-helper cells (%)	46	NS
T-helper cells (x 10^9/l)	46	NS
T-cytotoxic cells (%)	46	NS
T-cytotoxic cells (x 10^9/l)	46	NS
B-cells (%)	46	0.0366
B-cells (x 10^9/l)	46	0.0374
Natural killer cells (%)	46	NS
Natural killer cells (x 10^9/l)	46	NS

2. Lymphocyte Proliferation Response to PHA and Con A

Difference in value (day 2 – day 1)	N	P-value
PHA, 5 mg/l (cpm)	46	NS
PHA, 5 mg/l (cpm/1000 T cells)	46	NS
PHA, 10 mg/l (cpm)	46	NS
PHA, 10 mg/l (cpm/1000 T cells)	46	NS
Con A, 12 mg/l (cpm)	46	0.0361
Con A, 12 mg/l (cpm/1000 T cells)	46	NS
Con A, 25 mg/l (cpm)	46	NS
Con A, 25 mg/l (cpm/1000 T cells)	46	NS

3. Cytokine production by stimulated mononuclear cells:

Difference in value (day 2 – day 1)	N*	P-value
IL-1β (pg/ml supernatant)	40	NS
IL-2 (pg/ml supernatant)	18	NS
IL-6 (pg/ml supernatant)	16	NS

*Day 2 samples for IL-2 and IL-6 were determined only for n=16 and n=18 respectively. Therefore paired t test was done on that subsample.

APPENDIX J
SUMMARY OF DISCRIMINANT ANALYSIS FINAL MODELS FOR CLASSIFYING INDIVIDUALS INTO LOW AND HIGH RESPONDERS ON IMMUNE FUNCTION TESTS

Variables	Final models	BMI	Protein	Alb	Ft	TfR	TS	Hb	Hct	MCV	RDW	Plasma zinc	Serum vitamin B_{12}	Serum folate	Error rate
T cells and subsets															
Total T cells	1		x		x					x		x			37.2 %
	2		x		x					x					38.2 %
T helper cells	1	x	x					x	x			x			16.5 %
	2	x	x					x	x						27.7 %
T cytotoxic cells	1			x				x		x		x			25.4 %
	2		x					x		x		x			28 %
Mitogen proliferation															
PHA 5/1000 T cells	1		x		x			x	x		x				15.4 %
	2		x					x	x		x				16.5 %
PHA 10/1000 T cells	1		x			x		x	x	x					20.7 %
	2		x			x		x	x						21.6 %
Con A 12/1000 T cells	1		x		x			x	x			x			10.3 %
	2		x		x			x	x						15.9 %
Con A 25/1000 T cells	1		x		x			x				x			15 %
	2		x		x			x							28.6 %
Cytokines															
IL-2/1000 T cells	1		x		x								x		13.4 %
	2		x					x					x		19.2 %
IL-2/1000 T helper cells	1		x		x								x		11.1 %
	2		x					x					x		12.5 %
IL-1/1000 granulocytes	1		x			x			x				x	x	22.2 %
	2		x			x			x				x		25.3 %
IL-1/1000 (granulocytes + monocytes)	1		x			x			x				x	x	19.5 %
	2		x			x			x				x		23.1 %
IL-6/1000 granulocytes	1		x			x			x				x	x	18.9 %
	2		x			x			x				x		23.3 %
IL-6/1000 (granulocytes + monocytes)	1		x			x			x				x	x	17.5 %
	2		x			x			x				x		23.2 %

*Alb – albumin; Ft – serum ferritin; TfR – serum transferrin receptor; TS – transferrin saturation; Hb – hemoglobin; Hct – hematocrit; RDW – red cell distribution width

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PUBLICATIONS

Publications in Scientific Journals

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