

Markers to measure immunomodulation in human nutrition intervention studies†

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Normal functioning of the immune system is crucial to the health of man, and diet is one of the major exogenous factors modulating individual immunocompetence. Recently, nutrition research has focused on the role of foods or specific food components in enhancing immune system responsiveness to challenges and thereby improving health and reducing disease risks. Assessing diet-induced changes of immune function, however, requires a thorough methodological approach targeting a large spectrum of immune system parameters. Currently, no single marker is available to predict the outcome of a dietary intervention on the resistance to infection or to other immune system-related diseases. The present review summarises the immune function assays commonly used as markers in human intervention studies and evaluates their biological relevance (e.g. known correlation with clinically relevant endpoints), sensitivity (e.g. within- and between-subject variation), and practical feasibility. Based on these criteria markers were classified into three categories with high, medium or low suitability. Vaccine-specific serum antibody production, delayed-type hypersensitivity response, vaccine-specific or total secretory IgA in saliva and the response to attenuated pathogens, were classified as markers with high suitability. Markers with medium suitability include natural killer cell cytotoxicity, oxidative burst of phagocytes, lymphocyte proliferation and the cytokine pattern produced by activated immune cells. Since no single marker allows conclusions to be drawn about the modulation of the whole immune system, except for the clinical outcome of infection itself, combining markers with high and medium suitability is currently the best approach to measure immunomodulation in human nutrition intervention studies. It would be valuable to include several immune markers in addition to clinical outcome in future clinical trials in this area, as there is too little evidence that correlates markers with global health improvement.

Immune function: Marker: Diet: Human studies: Infections

Task and objectives

The major function of the immune system is to protect the body against infectious diseases. The immune system can be divided into innate and adaptive immunity. The immune system operates at the systemic as well as at the local level, which includes the

mucosal tissue such as in the upper airways and the gut. A fundamental characteristic of the immune system is that it involves multiple, functionally differing cell types, which permit a large variety of defence mechanisms. Assessing the status of the immune system and its functionality therefore requires a thorough methodological approach targeting a large spectrum of immune

Abbreviations: APC, antigen-presenting cell; CD, cluster of differentiation; DTH, delayed-type hypersensitivity; HLA, human leucocyte antigen; IFN, interferon; ILSI, International Life Sciences Institute; LPS, lipopolysaccharide; NK, natural killer; PBMC, peripheral blood mononuclear cell; PEM, protein–energy malnutrition; PG, prostaglandin; TH, T helper; TLR, toll-like receptor; URTI, upper respiratory-tract infection.

†A draft version of this review was extensively discussed with experts from the fields of nutrition, (clinical) immunology, mucosal immunology, gastroenterology and immunotoxicology during a Workshop, organised by the European branch of the International Life Sciences Institute (ILSI Europe), on ‘Markers to Assess the Impact of Nutrition on Immune Function in Man’ held in Vienna, Austria, 9–11 June 2004.

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system parameters. However, currently it is not possible to predict the cumulative effects of several small changes in immune system parameters on host resistance (Keil *et al.* 2001).

Nutrition is known to affect the immune system and thereby modulates resistance to infection (Chandra, 1991; Scrimshaw & SanGiovanni, 1997). At the single nutrient level, it has been shown that all immune functions rely on an adequate nutrient supply in order to function properly. Human trials have provided evidence that supplementation with single nutrients, as well as qualitative changes in certain macronutrients, affect specific immune functions even in well-nourished individuals. A major focus of current research is the role of specific food components or foods in enhancing immune system responsiveness to challenge with the aim of improving health and reducing disease risk. Target groups are the general population, as well as certain vulnerable groups with particular sensitivity towards infectious diseases.

The aim of the present review is to summarise existing knowledge on the quality of the markers commonly used to assess immune functions in healthy human subjects. This includes a description of the standard immune function assays used in human studies, their specificity, normal range and dynamics of change, their statistical validation, and their known correlation with clinical endpoints. The majority of human studies have looked at changes in systemic immunity and only a few studies have tried to measure the effect of dietary interventions on the gut immune system. Because experimental data indicate that diet affects the immune system associated with the intestinal tract (Roller *et al.* 2004), the review also includes current methods applied at the intestinal level. Most of the immune markers considered can be used in various subgroups of the population (infants, elderly, etc.) but the appropriate selection of markers depends on the objectives of individual studies (clinical studies, field work, etc.). The overall aim is to identify an appropriate set of relevant markers of immune functions that could be used to measure enhanced immune functions, including those of the gut immune system, in response to a nutritional intervention and to substantiate suitable markers for improved resistance to infection. The present review paper was prepared by the Expert Group on 'Nutrients and Immune Resistance to Infections' of the Nutrition and Immunity in Man Task Force of the European branch of the International Life Sciences Institute (ILSI Europe). A draft version of this review was extensively discussed with experts from the fields of nutrition, (clinical) immunology, mucosal immunology, gastroenterology and immunotoxicology during the ILSI Europe Workshop 'Markers to Assess the Impact of Nutrition on Immune Function in Man' held in Vienna, Austria, 9–11 June 2004.

Immune functions and health

Resistance to infection is strongly influenced by the effectiveness of the immune system in protecting the host against pathogenic micro-organisms. A comprehensive description of the human immune system can be found in many textbooks (e.g. Janeway *et al.* 2005). Immune function is influenced by genetic as well as environmental factors and thus there is some degree of variability in resistance to infection within the normal healthy adult population. Resistance to specific infections is also affected by previous exposure to the disease-causing pathogen or inoculation with vaccines used for immunisation. Vaccines contain dead or

attenuated pathogens that trigger immune responses including the development of specific memory without eliciting symptoms of disease that are associated with inoculation by wild-type pathogens.

Age is a critical factor in resistance to infection. Antigen-specific cellular and humoral immunity are central to the adaptive immune responses generated in the human adult. In contrast, the very young rely primarily on innate immunity although this component of the immune system is not as functionally developed in young children as it is in adults. Although many previous studies have demonstrated a marked decline in several aspects of immune function in the elderly, it is now recognised that some immune responses do not decline and can even increase with advancing age (Lesourd *et al.* 2002). Nowadays the influence of ageing on the immune system is generally described as a progressive occurrence of dysregulation, rather than as a general decline in function. Indeed, it has also been shown that many decreased immune responses that were previously attributed to the ageing process are actually linked to other factors such as poor nutritional status or an ongoing disease that is not clinically apparent (Lesourd *et al.* 2002).

The sex of the individual also affects immune function. In females, oestrogens and progesterone modulate immune function (Paavonen, 1994) and thus immunity is influenced by the menstrual cycle and pregnancy (Haus & Smolensky, 1999). Consequently, sex-based differences in responses to infection, trauma and sepsis are evident (Beery, 2003). Evaluation of immune responses must take into account sex differences in the study population as well as the menstrual cycle and hormonal treatment. Women are generally more resistant to viral infections and tend to have more autoimmune diseases than men (Beery, 2003). Oestrogens are generally immune-enhancing, whereas androgens, including testosterone, exert suppressive effects on both humoral and cellular immune responses. In females, there is increased expression of some cytokines in peripheral blood and vaginal fluids during the follicular phase of the menstrual cycle and with use of hormonal contraceptives (Brabin, 2002). In the luteal phase of the menstrual cycle, blood leucocyte counts are higher than in the follicular phase and the immune response is shifted towards a T helper (TH) 2-type response (Faas *et al.* 2000). In pregnancy, elevated levels of progesterone appear to suppress cell-mediated immune function and TH1 cytokine production and to enhance humoral immunity and TH2 cytokine production (Wilder, 1998).

Psychological stress is thought to influence immune function through autonomic nerves innervating lymphoid tissue and by stress hormone-mediated alteration of immune cell functions (Cohen *et al.* 1991). Stress hormones (particularly catecholamines and glucocorticoids) are potent modulators of immune function. Chronic psychological stress also appears to lower salivary IgA levels, evidenced by a transient decrease in the levels of salivary IgA in students under academic examination stress (Jemmott *et al.* 1983). The literature concerning the relationship between psychological stress and immunodepression is inconsistent, largely due to the numerous variables that need to be controlled. However, Cohen *et al.* (1991) carried out a well-controlled study (including controls for education, shared housing and personality differences) in which subjects were intentionally exposed to one of five respiratory viruses via nasal drops. The results indicated that psychological stress is associated with an increased risk of infection independent of the possibility of transmission, the

strain of administered virus and habitual physical activity. Psychological stress may also modify immune responses through the adoption of coping behaviours, e.g. increased alcohol consumption or smoking. Elevated levels of stress hormones also occur during strenuous exercise and it is well recognised that acute bouts of exercise cause a temporary depression of various aspects of immune function (e.g. neutrophil oxidative burst, lymphocyte proliferation, monocyte MHC class II expression) that lasts about 3 to 24 h after exercise depending on the intensity and duration of the exercise bout (Gleeson & Bishop, 1999). Periods of intensified training (over-reaching) lasting 7 d or more result in chronically depressed immune function and several surveys (e.g. Peters & Bateman, 1983; Nieman *et al.* 1990; Heath *et al.* 1991) indicate that sore throats and flu-like symptoms are more common in endurance athletes than in the general population.

It is well established that the general nutritional status of an individual modulates his or her immune functions. Both overnutrition that results in obesity (Samartin & Chandra, 2001) and undernutrition (Chandra, 1991; Scrimshaw & SanGiovanni, 1997) affect functions of innate and acquired immunity detrimentally. Further, obesity (BMI > 30 kg/m²) can be associated with chronic inflammation, resulting in increased plasma concentrations of C-reactive protein (CRP), IL-6, TNF- α and plasminogen activator inhibitor-1 (Dandona *et al.* 2004).

Particular aspects of the habitual diet including fat and protein intakes, multivitamin and mineral supplements and alcohol consumption exert a significant influence on immune function. Deficiencies of specific micronutrients are associated with an impaired immune response and with an increased susceptibility to infectious disease. If a nutrient supplement corrects an existing deficiency in an adult, then it is likely that a benefit to immune function will be seen. Indeed, many human and animal studies have demonstrated that adding the deficient micronutrient back to the diet will restore immune function and resistance to infection (Calder & Kew, 2002). What is far less clear is whether increasing the intakes of specific micronutrients above those recommended will improve immune function in a healthy well-nourished individual. There is also a danger of excessive supplementation of the diet with individual micronutrients. Excessive intakes of some micronutrients (e.g. vitamin E, Fe and Zn) impair immune function and increase susceptibility to infection (Chandra, 1984; Bogden *et al.* 1990; Sherman, 1992). Thus, for many micronutrients there is a limited range of optimum intake, with levels above or below this resulting in impaired immune function and/or other health problems (Calder & Kew, 2002).

Infectious diseases can affect the status of several nutrients in the body, thus setting up a vicious circle of undernutrition, compromised immunity and recurrent infection. Undernutrition is not a problem that is restricted to poor or developing countries. Undernutrition exists in developed countries especially among the elderly, premature babies, individuals with eating disorders, alcoholics and patients with certain diseases. Malnutrition was the leading cause of acquired immune deficiency before the appearance of the HIV and poor nutrition is also a major factor contributing to the progression of HIV infection.

In addition, several diseases that exist among the apparently well-nourished population have a strong immunological component. Examples of such diseases include asthma, atherosclerosis, cancer, Crohn's disease, myasthenia gravis, multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus and

food allergies, and it is now well recognised that the course of some of these can be influenced by diet. For some of these diseases, symptoms may be caused or aggravated by an inappropriately activated immune system. Although a primary function of the immune function is to destroy pathogenic micro-organisms, it can also damage body tissues. Usually the inflammation and tissue destruction that are associated with the mechanisms used to eradicate a pathogen are acceptable to the host and do not cause significant impairment of host function. However, in several diseases (e.g. rheumatoid arthritis) the tissue destruction by the activated immune system is substantial, long-lasting and harmful. It is because of the potentially damaging effects of the immune cells on body tissues that the system is very tightly regulated. Failure of these regulatory mechanisms can result in the full might of the immune system being inappropriately directed against the body's own tissues and in the development of chronic inflammatory or autoimmune diseases. Clearly, attempts to stimulate immune function by nutritional means are inappropriate in these conditions. The suppression of inappropriate immune activity may be desirable and there is some evidence that the anti-inflammatory and immunosuppressive effects of long-chain *n*-3 PUFA may be of use as a therapy for chronic inflammation and for disorders that involve an inappropriately activated immune response (Calder & Field, 2002).

Changes in immune function during life and their significance in adults, infants, the elderly and exercising people

Healthy adults

The introduction of an infectious agent into the body initiates an inflammatory response that augments that of the immune system. Acute inflammation increases local blood flow in the infected area and this coupled with augmented vascular permeability facilitates the entry of leucocytes and plasma proteins into the infected tissue. The immune response itself varies according to the nature of the infectious agent (parasitic, bacterial, fungal, viral) but a general response pattern is evident. Conserved molecular patterns on microbes are recognised by toll-like receptors (TLR) on macrophages and initiate intracellular signalling pathways that result in induction of co-stimulatory molecule expression and cytokine production. TLR appear to play an essential role in the activation of both innate and adaptive immunity (Schnare *et al.* 2001). Following ingestion of the micro-organism by the phagocytic macrophage, enzymes and oxidising agents are released from within the macrophage. The foreign proteins normally found on the micro-organism's surface are processed by the macrophage and incorporated into its own cell surface and are presented alongside MHC class II proteins. The antigen can now be presented to the other cellular immune components. TH cells (characterised by the expression of cluster of differentiation (CD) 4 on their surface) coordinate the response via TH1 and TH2 cytokine release to activate other immune cells. TH1 activation primarily promotes the actions of cytotoxic T cells, macrophages and non-specific natural killer (NK) cells which are responsible for cell-mediated immunity and are effective in the elimination of intracellular pathogens. TH1 cells can also stimulate the production of IgG1 and IgG3 by B cells. TH2 activation results in proliferation and stimulation of IgG4 and IgE production by B cells. Binding of the immunoglobulin to a specific antigen forms an antibody-antigen complex. This represents the

humoral (fluid) immune response and is an effective defence against extracellular pathogens present in the body fluids.

Whether humoral or cell-mediated immunity will dominate depends largely on the type of cytokines that are released by the activated TH cells. Cell-mediated immunity depends on a so-called TH1 profile of cytokines, including particularly interferon (IFN)- γ and IL-2. These cytokines activate macrophages and induce killer mechanisms that involve cytotoxic T cells. A TH2 profile includes mainly IL-4, IL-5 and IL-13, which are necessary for promotion of humoral immunity, IgE-mediated allergic reactions and activation of potentially tissue-damaging eosinophils. IL-4 and IL-13 primarily drive B cell differentiation to antibody production, while IL-5 stimulates and primes eosinophils (Cummings *et al.* 2004).

In recent years great efforts have been made to elucidate the mechanisms involved in the induction and regulation of a polarised cytokine profile characterising activated TH cell subsets. There is particularly great interest in the role of antigen-presenting cells (APC) in shaping the phenotypes of naïve T cells during their initial priming, partly because the differential expression level of various co-stimulatory molecules on activated and matured APC may exert a decisive impact (Liew, 2002). Thus, interaction of the CD28 receptor on T cells with CD80 on APC appears to favour TH1 differentiation, whereas interaction with CD86 appears to favour the TH2 phenotype. Certain cytokines secreted by the TH1 and TH2 cells that evolve act in an autocrine and reciprocally inhibitory fashion: IL-4 promotes TH2 cell expansion and limits proliferation of TH1 cells, whereas IFN- γ enhances growth of TH1 cells but decreases TH2 cell development. In fact, the cytokine microenvironment clearly represents a potent determinant of TH1/TH2 polarisation, with IL-4 and IL-12 as the initiating key factors, these being derived principally from innate immune responses during T cell priming. Activated APC are the main source of IL-12, whereas an early burst of IL-4 may come from NK cells, mast cells, basophils or already matured bystander TH2 cells (Liew, 2002).

Altogether, exogenous stimuli such as pathogen-derived products and the maturational stage of APC, as well as genetic factors, will influence differentiation into the TH1 or TH2 phenotype in addition to complex interactions between antigen dose, T-cell receptor engagement and MHC antigen affinities. Influential antigenic properties include the nature of the antigen, with bacteria and viruses promoting TH1 cell differentiation and helminths the TH2 subset. TH2 differentiation also appears to be promoted by small soluble proteins characteristic of allergens.

Although it is somewhat of an oversimplification, the TH1 response can be seen as the major promoter of cell-mediated reactions that provide effective defence against intracellular pathogens (i.e. viruses and bacteria that can enter host cells or are phagocytosed). In contrast, the TH2 response primarily activates humoral immunity and the antibodies produced are only effective against pathogens in the extracellular fluids. As mentioned previously, TH1- and TH2-type responses are cross-regulatory, and the TH1/TH2 cytokine balance is also influenced by regulatory TH3 cells (Maloy & Powrie 2001), which may secrete the suppressive cytokines IL-10 and transforming growth factor (TGF)- β and thus exert a dampening effect directly on innate immune mechanisms (Maloy *et al.* 2003).

In healthy normal adults, small decreases or increases in single selected markers of immune function may not be clinically important. There are two main reasons for this. First, there is a

considerable degree of redundancy in the immune system, such that a small change in the functional capacity of one component of immune function may be compensated for by a change in the functional capacity of another. Second, there may be a certain amount of excess capacity in some aspects of immune function, particularly for those functions that are assessed using *in vitro* challenges using a high concentration of stimulant. Thus, it cannot be stated with any degree of certainty that small increases in one or more aspects of immune function will alter an individual's susceptibility to infection. Indeed for many aspects of immune function (e.g. blood neutrophil count and oxidative burst activity), it is not even known if the normal variation seen in the healthy adult population is a factor that influences the ability to fight infections. A more substantial increase in one or more aspects of immune function is probably more likely to reduce infection risk, although, of course, infection risk also depends on the degree of exposure to pathogens and the experience of previous exposure. However, for some immune cell functions a sufficiently large variation or change has been related to improved host defence. For example, some studies indicate that susceptibility to infections and cancer is greater in individuals who possess low NK cell activity compared with individuals with moderate to high NK cell activity (Levy *et al.* 1991; Imai *et al.* 2000; Ogata *et al.* 2001). Increasing several aspects of immune function would be expected to convey a more effective immune protection than an increase in just one aspect of immune function. It should be borne in mind that the relationship between a specific aspect of immune function and intake of a specific nutrient will not be the same for all immune cell functions. Indeed, it is entirely possible that the intake of a particular nutrient that produces optimum function in one aspect of immunity might result in sub-optimum function in another. Hence, it is important that studies on the effects of foods or food components on immunity measure a wide range of different immune markers. Ultimately, the real test of the efficacy of a food or food component that claims to improve immune function is a change in the incidence of infectious episodes or the severity or duration of symptoms of infection as this is the outcome of greatest clinical significance. When this can be confirmed, the measurement of a change in one or more aspects of immune function may provide information on the likely mechanism of the dietary intervention.

Infants (paediatric immunology)

The newborn child is immunologically competent. For example, the paediatric immune system handles infection and responds appropriately to immunisation. However, while many of the immune mechanisms that are present in the adult are also found in the child, there are a number of differences. These differences are due to two main interrelated factors. The first is the development of the immune system: certain aspects of the adaptive and innate immune systems are not fully functional at birth and develop thereafter. The second is a consequence of the low exposure to antigen until after birth. Thus, instruction of the acquired immune system, by definition, is incomplete at birth.

The present section highlights only important aspects of the paediatric immune system; for greater coverage, the reader is referred to larger texts (Spirer *et al.* 1993; Wolf, 2004). Innate immune mechanisms in infants are similar to those of adults because they do not require instruction. Nevertheless, there are

some differences in innate immunity during ontogeny shown in species other than man.

Of the various components of the immune system that change after birth, the functions of the B lymphocyte lineage show the greatest alterations. Ig levels in the circulation of the newborn infant are low, apart from IgG transferred *in utero* from the maternal circulation. IgA is almost undetectable and IgM levels are also low, but do increase rapidly with an antigenic challenge such as neonatal infections. IgM levels above 0.2 mg/ml in cord blood suggest a congenital infection.

Because cord blood is easily obtained, the immune system in the immediate newborn period has been well described. Of the mononuclear cells in human cord blood, 80% are T lymphocytes, 10% are B cells and 10% are monocytes. The T lymphocytes express a range of cytokines that are similar to that of a TH2 response. Only during the first year of life does the predominant response of the T cell become that of a TH1. The development of the thymus and lymphoid tissue follows a pattern quite dissimilar to that of any other human organ. Most organs have their greatest period of growth in the early neonatal period with decreasing rates of growth thereafter. Organs that are dependent on puberty, such as those necessary for reproduction and growth, have an increase soon after the end of the first decade of life. The lymphoid system, however, increases in size faster than the body until around 7 years of age. It then becomes much smaller, reaching adult levels by the age of 12 years. These large increases in lymphoid tissue are the cause of well-recognised changes in the child such as large tonsils and adenoids (occasionally giving rise to problems with the upper airway) and also an increase in lymphoid nodules seen throughout the intestine at endoscopy.

The childhood period is also that in which most immunisations are given. The use of immunisations has revolutionised the practice of childhood medicine. The adequate response to immunisations has resulted in the virtual eradication and/or elimination of serious sequelae from measles, polio, whooping cough and *Haemophilus* infections.

Elderly

Ageing is associated with important but variable changes in immune responses. Some immune responses, such as blood IgG or IgA levels, lymphocyte proliferation and TH1 responses, decline with ageing, while others, such as prostaglandin (PG) E₂ production or TH2 responses, increase (Lesourd *et al.* 2002). These changes have been reported to be due to either hormonal modifications throughout life (i.e. decline in thymic hormones after puberty) or to accumulating antigenic pressure during the life span. These changes are nowadays described as a progressive occurrence of immune dysregulation that leads to decreased cell-mediated immune responses and relatively preserved antibody responses. Aged individuals are more sensitive to intracellular infections than younger adults (Pawelec *et al.* 2002). In addition, non-specific immunity appears to be less affected by the ageing process but induces a longer inflammatory process in the elderly.

Cell-mediated immune responses decline with ageing. Decline in thymic functions leads to a progressive decline in CD45RA (naïve) T cell subsets. Simultaneously CD2⁺CD3⁻ subsets (NK cells) increase, showing another example of inverse evolution of immune responses (here cell subsets) with age.

Antigenic pressure throughout life leads to increases in memory T cells (many of which express CD45RO) and a decrease

in naïve T cells (many of which express CD45RA; Cossarizza *et al.* 1992). Both changes affect T cell functions with a progressive decline in both lymphocyte proliferation and IL-2 synthesis, which are strongly associated with higher prevalence of infectious diseases in most elderly persons, although those selected for extreme good health (e.g. using the SENIEUR protocol; Ligthart *et al.* 1984) show similar levels of T cell proliferation and IL-2 production as healthy young people. Thus, these changes are less important in the very healthy elderly (self-sufficient, free-living with no apparent disease and no decline in cognitive functions) in whom they are significant only at very old age (>90 years). However, they occur sooner in the less healthy frail elderly, indicating that disease-associated decreased immune responses play an important role in the decline in cell-mediated immunity. This may be more important than the ageing process *per se* (Mazari & Lesourd, 1998). Ageing is also associated with a decline in CD8⁺ cytotoxic T cells while the CD4⁺ subset seems to be preserved as long as nutritional status is 'normal'. However, an inverted CD4:CD8 ratio due to an increase in the number of dysfunctional CD8 cells predominantly specific for cytomegalovirus epitopes is commonly observed in the very elderly and is predictive of incipient mortality (Pawelec *et al.* 2004). The TH1:TH2 ratio also declines with age and this has been related to accumulation of antigenic pressure throughout life (Cakman *et al.* 1996). These changes may explain the decline in CD8⁺ cell cytotoxic functions, which are TH1-dependent, and the relatively preserved B cell functions, which are TH2-dependent. However, decreased numbers of B cells also contribute to predicting incipient mortality in longitudinal studies. These changes may be quantified by measurement of *in vitro* cytokine release in lymphocyte cultures (i.e. decreases in TH1 cytokines such as IL-2 or increases in TH2 cytokines such as IL-4, IL-5 or IL-13). Nevertheless, contradictory results have been reported: for example, IFN- γ , a TH1 cytokine, has been reported to decrease, to remain unchanged or even to increase with ageing. Therefore, this general picture of TH1/TH2 changes with ageing must still be viewed with some caution. For an excellent survey of the available data and pitfalls in their generation and interpretation, see Gardner & Murasko (2002).

Antibody responses, encompassing IgA, IgG or IgM responses after antigenic exposure (such as vaccination), are comparable in healthy elderly and younger adults. This may be related to the relative increase in TH2 responses with ageing that boost IgA and IgG production. Nevertheless, even though the antibody level does not change or even rises, antibody affinity declines with ageing. This has been associated with progressive decreases in the CD5⁻ B cell subset that is responsible for the high-affinity antibody while the CD5⁺ subset, which produces lower-affinity antibody, increases (Weksler, 1995). In addition, a high level of anti-idiotypic antibody production has been described after inoculation with tetanus vaccine in aged individuals (Arreaza *et al.* 1993). These changes in antibody affinity may partly explain the lower protection sometimes observed after vaccination in healthy aged individuals.

Monocyte functions, including antigen processing and presentation as well as cytokine release (TNF- α , IL-1, IL-6) are unchanged or even increased in the elderly (Lesourd, 1999). High resting serum IL-6 levels have often been reported in the apparently healthy elderly population, a phenomenon that has never been described in younger healthy individuals. This has been linked to a permanent activation of monocytes, which is

associated with higher PGE₂ and free radical production (Hayek *et al.* 1997). Such a phenomenon is detrimental for cell-mediated immunity since PGE₂ is a strong inhibitor of T cell functions, particularly in aged persons. This represents an important age-related dysregulation of the immune system. Inflammatory processes are always of long duration in the elderly. In fact, a longer period of hormone secretion, as measured by the rise in the plasma cortisol level (Sapolsky *et al.* 1986), after stress challenge, is a general phenomenon in aged rats. These longer inflammatory processes lead to higher use of body nutrient reserves in stressed aged individuals. This is particularly dangerous in the elderly person since he or she is no longer able to completely restore depleted nutritional body reserves, particularly muscle proteins (Lesourd, 1999). Therefore any stress, through a longer activation of the immune system, pushes the elderly towards undernutrition and a more fragile physical state.

Undernutrition exerts a strong effect on immune responses in the elderly. Protein–energy malnutrition (PEM) is always associated with lower immune responses and this is observed for all types of immunity: cell-mediated immunity, antibody responses as well as innate immunity. This effect is strongly correlated with the severity of PEM. Undernourished elderly individuals are therefore at high risk for infectious diseases. The effects of micronutrient deficiencies are more often observed in aged individuals than in younger people (Lesourd, 2000). Nutritional supplementation, whether macronutrient energy supplements in PEM or micronutrient supplements in micronutrient deficiency states, usually leads to increased immune responses (Lesourd *et al.* 1998). In addition, while vitamin E deficiency is not commonly reported in the elderly, cell-mediated immune responses are increased after vitamin E supplementation (Meydani *et al.* 1997) showing that vitamin E needs may be higher in aged individuals than the current recommendations specify. Vitamin E supplementation is associated with decreases in PGE₂ and free radical production by monocytes, showing that the permanent activation of monocytes is detrimental to immune responses in the elderly.

The dysregulation of immune responses observed in the elderly is probably due to cumulative pressure on the immune system throughout life, driving T cell differentiation towards a limited repertoire of dysfunctional T cell memory responses. This, coupled with decreased thymic output of naïve T cells, as well as age-associated compromised function of naïve cells produced earlier in life, results in increased susceptibility of the elderly to challenge by new pathogens. Undernutrition, whatever its type, adds another detrimental factor to immune responses, the elderly being particularly susceptible to nutritional factors. Protection against permanently increased free radical production may be an effective way to boost immune responses in the elderly.

Exercise

Athletes engaged in heavy training programmes, particularly those involved in endurance events, appear to be more susceptible than the sedentary population to infection. For example, according to some surveys (e.g. Peters & Bateman 1983; Nieman *et al.* 1990; Heath *et al.* 1991) sore throats and flu-like symptoms are more common in athletes than in the general population and, once infected, colds may last for longer in athletes. There is some convincing evidence that this increased susceptibility to infection arises due to a depression of immune system function

(for detailed reviews see Shephard, 1997; Gleeson & Bishop, 1999; Mackinnon, 1999).

The circulating numbers and functional capacities of leucocytes may be decreased by repeated bouts of intense prolonged exercise. The reason is probably related to increased levels of stress hormones during exercise and entry into the circulation of less mature leucocytes from the bone marrow. Falls in the blood concentration of glutamine have also been suggested as a possible cause of the immunodepression associated with heavy training, although the evidence for this is less compelling. Inflammation caused by muscle damage may be another factor. Also, during exercise there is an increased production of reactive oxygen species and some immune cell functions can be impaired by an excess of free radicals (Niess *et al.* 1999).

During exercise exposure to airborne pathogens is increased due to the higher rate and depth of breathing. An increase in gut permeability may also allow increased entry of gut bacterial endotoxins into the circulation, particularly during prolonged exercise in the heat. Hence, the cause of the increased incidence of infection in athletes is likely to be multifactorial: a variety of stressors (physical, psychological, environmental, nutritional) can depress or suppress immune function and these effects together with increased exposure to pathogens can make the athlete more susceptible to infection.

The relationship between exercise and susceptibility to infection has been modelled in the form of a 'J' curve (Nieman, 1994). This model suggests that while engaging in moderate activity may enhance immune function above sedentary levels, excessive amounts of prolonged high-intensity exercise induce detrimental effects on immune function. However, although the literature provides strong evidence in support of the latter point (Nieman, 1994; Pyne, 1994; Pedersen & Bruunsgaard, 1995; Shephard, 1997; Gleeson & Bishop, 1999; Mackinnon, 1999), relatively little evidence is available to suggest that there is any clinically significant difference in immune function between sedentary and moderately active persons. Thus, it may be more realistic to 'flatten' out the portion of the curve representing this part of the relationship. Recently Matthews *et al.* (2002) reported that the regular performance of about 2 h of moderate exercise per d was associated with a 29% reduction in risk of picking up an upper respiratory-tract infection (URTI) compared with a sedentary lifestyle. In contrast, it has been reported that there is a 100–500% increase in risk of picking up an infection in the weeks following a competitive ultra-endurance running event (Nieman *et al.* 1990; Peters *et al.* 1993, 1996).

Acute effects of exercise on immune function. A single, acute session of prolonged strenuous exercise has a temporary depressive effect on immune function and this has been associated with an increased incidence of infection. For example, both Peters & Bateman (1983) and Nieman *et al.* (1990) have described a substantially higher (two- to six-fold) frequency of self-reported symptoms of URTI in athletes who completed long-distance foot races compared with control runners who did not compete in the events. An acute bout of physical activity is accompanied by responses that are remarkably similar in many respects to those induced by infection, sepsis or trauma (Northoff *et al.* 1998; Gleeson & Bishop, 1999): there is a substantial increase in the number of circulating leucocytes (mainly lymphocytes and neutrophils), the magnitude of which is related to both the intensity and duration of exercise. There are also increases in the plasma concentrations of various substances that are known

to influence leucocyte functions, including inflammatory and anti-inflammatory cytokines such as TNF- α , IL-1 β , IL-6, IL-10, macrophage inflammatory protein-1 and IL-1-receptor antagonist, acute-phase proteins such as CRP and activated complement fragments. The large increases in plasma IL-6 concentration observed during exercise can be entirely accounted for by release of this cytokine from activated muscle fibres (Steensberg *et al.* 2000). However, IL-6 production by monocytes (Starkie *et al.* 2001) and IL-2 and IFN- γ (but not IL-4) production by T lymphocytes are inhibited during and for several hours after prolonged exercise (Northoff *et al.* 1998; Gleeson, 2004). These cytokine changes suggest a shift in the TH1/TH2 balance towards a TH2 response, which would be expected to decrease host defence against intracellular pathogens.

Hormonal changes also occur in response to exercise, including rises in the plasma concentration of several hormones (e.g. adrenaline, cortisol, growth hormone and prolactin) that are known to have immunomodulatory effects. Phagocytic neutrophils appear to be activated by an acute bout of exercise, but show a diminished responsiveness to stimulation by bacterial lipopolysaccharide (LPS; including both reduced oxidative burst and diminished degranulation responses) after exercise, which can last for many hours (Pyne, 1994; Robson *et al.* 1999). Acute exercise temporarily increases the number of circulating NK cells but following exercise NK cell numbers decline to less than half of normal levels for a couple of hours; normal resting values are usually restored within 24 h (Shephard & Shek, 1999). NK cell cytolytic activity (per cell) falls after exercise and if the exercise is both prolonged and vigorous, the decrease in NK cell counts and cytolytic activity may begin during the exercise session (Shephard & Shek, 1999). During recovery from exercise, lymphokine-activated killer cell numbers and activity also fall below pre-exercise levels. Acute exercise has been shown to diminish the proliferative response of lymphocytes to mitogens (Mackinnon, 1999) and decrease the expression of an early activation marker (CD69) in response to stimulation with mitogen (Ronsen *et al.* 2001). When the exercise bout is strenuous and very prolonged (>1.5 h), the number of circulating lymphocytes may be decreased below pre-exercise levels for several hours after exercise and the T lymphocyte CD4⁺:CD8⁺ ratio is decreased (Berk *et al.* 1986; Pedersen & Bruunsgaard, 1995).

APC function is also affected by exercise: exercise-induced reductions in macrophage MHC class II expression and antigen-presenting capacity have been documented (Woods *et al.* 2000). Both memory (CD45RO⁺) and naïve (CD45RA⁺) T cells increase temporarily during exercise, but the CD45RO:CD45RA ratio tends to increase due to the relatively greater mobilisation of the CD45RO⁺ subset (Gannon *et al.* 2002; Lancaster *et al.* 2003a). Following prolonged strenuous exercise the production of Ig by B lymphocytes is inhibited and delayed-type hypersensitivity (DTH) responses (as measured using the CMI Multitest[®] kit) are diminished (Bruunsgaard *et al.* 1997). After prolonged exercise, the plasma concentration of glutamine has been reported to fall by about 20% and may remain depressed for some time. These changes during early recovery from exercise would appear to weaken the potential immune response to pathogens and have been suggested to provide an 'open window' for infection, representing the most vulnerable time period for an athlete in terms of their susceptibility to contracting an infection (Pedersen & Bruunsgaard, 1995). A new and potentially important finding is that following a prolonged bout of strenuous exercise the

expression of some TLR on monocytes is decreased (Lancaster *et al.* 2003c). Furthermore, this is associated with decreased induction of co-stimulatory molecules and cytokines following stimulation with known TLR ligands. These effects may represent a mechanism through which exercise stress impairs immune function and increases susceptibility to infection.

Chronic effects of exercise training on immune function. Chronic exercise (i.e. exercise training) also modifies immune function, with most changes on balance suggesting an overall decrease in immune system function, particularly when training loads are high (Gleeson & Bishop, 1999). Circulating numbers of leucocytes are generally lower in athletes at rest than in sedentary people, although there is a weak suggestion of a slightly elevated NK cell count and cytolytic action in trained individuals (Shephard & Shek, 1999). A low blood leucocyte count may arise from the haemodilution (expansion of the plasma volume) associated with training, or may represent increased apoptosis or altered leucocyte kinetics including a diminished release from the bone marrow. Indeed, the large increase in circulating neutrophil numbers that accompanies a bout of prolonged exercise could, over periods of months or years of heavy training, deplete the bone marrow reserve of these important cells. Certainly, the blood population of these cells seems to be less mature than that found in sedentary individuals (Pyne, 1994) and the phagocytic and oxidative burst activities of stimulated neutrophils have been reported to be markedly lower in well-trained cyclists than in age- and weight-matched sedentary controls (Blannin *et al.* 1996). Levels of secretory Ig such as salivary IgA are lower in athletes engaged in heavy training (Gleeson, 2000), as are T lymphocyte CD4⁺:CD8⁺ ratios and *in vitro* mitogen-stimulated lymphocyte proliferation responses (Verde *et al.* 1992; Lancaster *et al.* 2003b). However, exercise training in healthy young adults does not appear to have an effect on the initiation of a specific antibody response to vaccination or DTH responses as measured with the CMI Multitest[®] kit (Bruunsgaard *et al.* 1997). Thus, with chronic periods of heavy training, several aspects of both innate and acquired immunity are depressed.

There are several possible causes of the diminution of immune function associated with heavy training. One mechanism may simply be the cumulative effects of repeated sessions of intense exercise with the consequent elevation of stress hormones, particularly glucocorticoids such as cortisol, causing temporary immunodepression. It is known that both acute glucocorticosteroid administration (Moynihan *et al.* 1998) and exercise cause a temporary inhibition of IFN- γ production by T lymphocytes and it has been suggested that this may be an important mechanism in exercise-induced depression of immune cell functions (Northoff *et al.* 1998). When exercise is repeated frequently there may not be sufficient time for the immune system to recover fully. Furthermore, plasma glutamine levels can change substantially after exercise and may become chronically depressed after repeated short periods of prolonged strenuous training (Shephard, 1997). Complement activation also occurs during exercise and a diminution of the serum complement concentration with repeated bouts of exercise, particularly when muscle damage is incurred, could also contribute to decreased innate immunity in athletes (Smith *et al.* 1990); well-trained individuals have a lower serum complement concentration compared with sedentary controls (Mackinnon, 1999).

In summary, acute short periods of exercise cause a temporary depression of various aspects of immune function (e.g. neutrophil

respiratory burst, lymphocyte proliferation, monocyte MHC class II expression) that lasts approximately 3 to 24 h after exercise depending on the intensity and duration of the exercise bout. Periods of intensified training (over-reaching) lasting 7 d or more result in chronically depressed immune function. Improvements in immune function in athletes as a result of consumption of a nutrient or a specific food could therefore be linked to: (i) an attenuation of the temporary immunodepression following a standardised session of exercise; (ii) an improvement in one or more aspects of immune function in the resting state; or (iii) both (i) and (ii).

Experimental design

The immune system is affected by a variety of subject-specific and technical factors, which, in an ideal study design, should be strictly controlled in order to reduce the variation in the outcome of immunological measurements (Table 1). In practice, not all factors can be controlled at the same time. In addition, ethical constraints may restrict the use of specific markers in certain populations. Subjects enrolled in human intervention studies should have a defined age range, since immune functions in the elderly can be decreased compared with young subjects, especially when nutrient intakes are low (Lesourd *et al.* 2002). The sex of the subjects being studied further affects immune functions through endogenous oestrogenic effects (Paavonen, 1994; Bouman *et al.* 2004). In addition, endogenous hormones during the menstrual cycle in female subjects, and exogenous hormones in the form of contraceptives or of hormone replacement therapy, consistently affect immune functions such as cytokine production (Haus & Smolensky, 1999), which requires female subjects to be classified as premenopausal (with and without contraceptives) or postmenopausal (with or without hormone replacement therapy). Short-term interventions starting at different phases of the menstrual cycle may also modulate the outcome of the study. BMI is another subject-specific factor with an impact on immune functions (Samartin & Chandra, 2001; Dandona *et al.* 2004). A study design accepting a wide range of BMI values may include study subjects with obesity-associated inflammation, which may interfere with the immunomodulating effects of the dietary intervention.

The background diet during the intervention is an important subject-specific factor often neglected in human intervention studies. It determines the general nutritional status of study subjects and thereby modulates their immune status. Including subjects with minor nutrient deficiencies on one hand or

subjects using daily multivitamin and mineral supplements on the other may severely influence the immunological impact of the intervention. Further, alcohol and probiotic consumption as well as the level of physical exercise and of smoking all affect immune functions and have to be controlled properly (Watzl & Watson, 1992; Gill & Cross, 2002; Petersen & Pedersen, 2002; Zeidel *et al.* 2002). The individual phenotype determines in another way the functional status of immune cells. For example, within a population of healthy individuals, NK cell activity can be reproducibly defined as low or high. Therefore, random allocation of individuals with high and low NK cell activity to different treatment groups is highly necessary in studies focusing on this measure of immune function, in order to avoid statistically significant pre-study differences in NK cell activity from occurring. In general the study should contain evidence that parameters of interest have been adequately randomised. Finally, the absence of infections as well as immune system-related diseases in study subjects is a fundamental prerequisite in nutritional immunology studies. Based on the assessment of these subject-specific factors, subjects should be properly matched.

Technical factors are more easily controlled and standardised than are subject-specific factors. First, the appropriate study population and the type of controls have to be identified. In some cases, study subjects could be their own controls, while in other cases a proper selection of controls should be included. Due to the circadian rhythm of immune cell activities (Haus & Smolensky, 1999), the timing of the blood collection and the fasting period before blood collection have to be standardised. Typically, blood is collected between 07.00 and 10.00 hours in the morning after an overnight fast. Seasonal variations due to environmental factors (differences in the length of the daily light and dark spans, climate, exposure to antigens, diet) may further affect immune functions (Haus & Smolensky, 1999; Nelson, 2004). The use of depletion or run-in periods prior to a dietary intervention study and appropriate washout periods in cross-over studies are further factors affecting variability. For example, a study investigating the role of different carotenoids on immune functions using a carotenoid depletion period before the beginning of the carotenoid supplementation observed enhanced mitogenic proliferative responsiveness of blood lymphocytes (Kramer & Burri, 1997), while the same carotenoid supplement had no significant effect on this function in subjects with normal plasma carotenoid profiles at baseline (Cross *et al.* 1998). The length of the intervention period can also modify the immunological outcome and the

Table 1. Confounding subject-specific and technical factors modulating immune function in human intervention studies

Subject-specific factors	Technical factors
Age	Selection of study population and appropriate controls
Sex (hormones, menstrual cycle)	Time of sample collection (circadian rhythm)
BMI	Season
Background diet before and during intervention (e.g. probiotic consumption, micronutrient supplementation)	Time since last meal (fasting period)
Physical exercise	Use of depletion/washout periods
Smoking	Length of intervention period
Genetics (low/high responder)	Appropriate selection of immune markers
Presence of infections or other diseases	
Psychological stress	
Sleep deprivation	
Alcohol, drug and medications	
Vaccination and infection history	

optimal time point to measure dietary effects on the immune system is often difficult to define. As an example, the intake of probiotics temporarily changes the microbial balance in the intestinal tract, which concurrently may initiate an immune response. However, one cannot exclude the possibility that the long-term intake of probiotics over months could result in adaptation and previous changes in immune functions may no longer be measurable. Finally, any dietary intervention should take into account the bioavailability of the relevant nutrient or food component by measuring its concentration or those of its metabolites in blood, urine or faeces and its interactions with other nutrients. The issue of the appropriate selection of immune assays will be discussed later.

Assessment of markers

Markers to assess immune function in human studies range from the whole organism level to the (sub)cellular, mechanistic level. Clinical endpoints such as mortality and morbidity from (common) infections reflect the overall balance between pathogen exposure and the integrated host defences and as such provide the most relevant indication of the ability to cope with common pathogens. However, natural exposure to pathogens is uncontrolled and unpredictable. This can be overcome experimentally by controlled exposure to vaccines comprising killed or attenuated micro-organisms that trigger *in vivo* immune responses. Such responses provide valuable information on the ability to respond to a 'model infection'. At the next level, individual aspects of innate and acquired immune function can be assessed *ex vivo*; i.e. using *in vitro* assays following *in vivo* dietary manipulation. Clearly, the clinical relevance of changes in these markers is less clear, but the sensitivity to detect differences may be better and results can provide important mechanistic information that can help in the generation of research hypotheses. Finally, circulating factors (i.e. total Ig, complement proteins, acute-phase proteins, cytokines and cytokine receptors) and cells (leucocytes and lymphocyte subsets) can be measured in blood/serum. These are not functional measures in that they are not indicative of a response to a controlled experimental stimulation of the immune system. Instead they are reflective of spontaneously ongoing responses *in vivo*. Table 2 provides an overview of the technical characteristics of the immune parameters most commonly used in human nutritional immunology studies. Table 3 provides additional details of the assays frequently used to measure these immune parameters.

A further issue relates to the storage of samples. While fresh cells should always be the first choice, for technical reasons it may sometimes be more practical to work with cryopreserved cells. Studies have shown that assessment of lymphocyte subsets and measurements of NK cell cytotoxicity and lymphocyte proliferation can be performed using cryopreserved cells (Jewett *et al.* 1976; Fujiwara *et al.* 1986; Whiteside *et al.* 1990; Tollerud *et al.* 1991; Allsopp *et al.* 1998). However, although most studies did not observe significant differences between fresh and cryopreserved cells, the outcome for some individuals might differ significantly between fresh and cryopreserved cells for unknown reasons. The effect of cryopreservation on lymphocyte proliferation depends on the stimulus used to activate the lymphocytes. There is also a risk that subsets of cells can be selectively lost (Jewett *et al.* 1976).

In vivo integrated responses

Immune response to vaccines

Although some trials have been published in which subjects were deliberately infected with pathogens such as rhinoviruses (Broadbent *et al.* 1984; Turner & Cetnarowski, 2000; Turner *et al.* 2000), *Shigella* (Tacket *et al.* 1992) or enterotoxigenic *Escherichia coli* (Bovee-Oudenhoven *et al.* 2003), such approaches have ethical constraints. Generally, it is more feasible to use vaccines with killed or attenuated pathogens as model infections. Vaccines trigger *in vivo* immune responses without eliciting symptoms of disease that would result from inoculation with live pathogens. Specific immune responses to vaccines that are part of a national vaccination schedule can therefore be used as *in vivo* indicators of the integrated response to those vaccines. Alternatively, one or more selected vaccinations can be integrated into the design of a study. In this case, different types of vaccines can be used to target selective aspects of *in vivo* immune responses. For instance, polysaccharide vaccines such as *Pneumococcus* initiate T cell-independent B cell responses, whereas first exposure to restricted-use vaccines such as hepatitis B elicits primary T cell-dependent responses. Secondary or subsequent exposure using vaccines to frequently occurring infections such as influenza or those used in vaccination programmes such as tetanus or diphtheria can be used to indicate recall (memory) responses.

Responses to vaccines are typically assessed as increased concentrations of vaccine-specific antibodies in serum or plasma that are measured by either ELISA or pathogen neutralisation assays. Responsiveness of vaccine-specific B cells can also be assessed as *ex vivo* secretion of vaccine-specific antibodies following stimulation with vaccine antigen. In addition, cell-mediated responses to certain vaccines have been assessed as a DTH response or as lymphocyte proliferation or cytokine production following *ex vivo* stimulation of lymphocytes with vaccine antigen (Leroux-Roels *et al.* 1994; Fletcher & Saliou, 2000; Wiedermann *et al.* 2000). Combination of different assays provides the opportunity to obtain more detailed information on the response elicited. Moreover, repeated measures can provide information on the kinetics of the dynamic response to a vaccine and can be used not only to assess the initial response to vaccination but also to evaluate the persistence of the antibody titre some months later. The latter is clinically important as, for example, an influenza vaccine given in early autumn would need to maintain high titres for 6 months to give protection throughout the influenza season. For other vaccines such as hepatitis and tetanus toxoid it is desirable to maintain high antibody titres for several years.

As vaccination elicits specific memory, volunteers can be admitted only once to a study that includes a certain vaccination. Also, responses can only be compared between study groups, precluding intra-individual analysis of the impact of intervention and use in trials with a cross-over design. In some cases, it may be necessary to stratify for baseline vaccine-specific immunity as this can vary largely within a population due to prior vaccinations or natural infections. The between-subject variability in response to vaccination is normally also quite high. The period between vaccination and the plateau phase of the response ranges from about 3 weeks (single-dose vaccines) to several months (hepatitis B), indicating that the study period should encompass at least this period. Responses to vaccination are widely used markers of immune function and provide high-quality information on the

Table 2. Technical and practical characteristics of selected immune function parameters

Parameter	Technical characteristics				Variability		Remarks
	Method	Equipment	Methods	Costs per sample	Need for standardisation	Within subjects	
Response to vaccination Delayed-type hypersensitivity	ELISA Skin test	Reader CMI Multitest® (no longer available)	Quantitative Semi-quantitative	Reasonable High	Normal Normal	N/A Low	High High Depends on vaccine
Phagocytosis	Flow cytometry	Mantoux method Flow cytometer	Semi-quantitative Quantitative	Reasonable Reasonable	High Normal	< 15% < 15%	10–20% 10–20% Variability granulocytes < monocytes Variability granulocytes < monocytes
Oxidative burst	Flow cytometry	Flow cytometer	Quantitative	Reasonable	Normal	< 15%	10–20% Up to 100% High High Depends on cytokine
Degranulation NK or LAK cell function	ELISA 51Cr release Flow cytometry Flow cytometry	Reader γ-Counter Flow cytometer Flow cytometer	Quantitative Quantitative Quantitative Quantitative	Reasonable High Reasonable High	Normal High Normal High	< 15% Low < 10% 10–25%	Up to 100% High High 35–60%
Cytokine production by monocytes, lymphocytes or whole blood	ELISA	Reader	Quantitative	High	High	5–10%	Up to 20-fold Variability whole blood < PBMC
Intracellular cytokines	RT-PCR	Thermal cycler	Semi-quantitative	Reasonable	High	< 10%	Low
Plasma cytokine concentrations	Real-time PCR	Thermal cycler	Quantitative	Reasonable	High	Low	Low
Eicosanoid concentration or production	Flow cytometry ELISA ELISA	Flow cytometer Reader Reader	Quantitative Quantitative Quantitative	High High High	Normal Normal Normal	Low Low	High High
Lymphocyte proliferation	Chromatography [3H]thymidine ELISA	GC/MS or HPLC/MS β-Counter Reader	Quantitative Quantitative Quantitative	High High Reasonable	Normal High Normal	10–40% < 10%	25–50% Up to 100% Depends on stimulus Variability net counts < stimulation index (ratio of stimulated to unstimulated counts)
Expression of markers of cellular activation	Flow cytometry Flow cytometry	Flow cytometer Flow cytometer	Quantitative Quantitative	Reasonable Reasonable	Normal Normal		
Ratio of CD45RO: CD45RA cells	Flow cytometry	Flow cytometer	Quantitative	Reasonable	Normal		
Leucocyte subsets	Flow cytometry	Flow cytometer	Qualitative/ quantitative	Reasonable	Normal	< 10%	10–50% Depends on subset (NK variable) Variability relative < absolute counts
GALT plasma cell function	ELISA RIA	Reader γ-Counter	Quantitative Quantitative	Reasonable Reasonable	Normal Normal		
Salivary IgA concentration	ELISA	Reader	Quantitative	Reasonable	Normal	15–20%	Up to 6-fold

NK, natural killer; LAK, lymphokine-activated killer; GALT, gut-associated lymphoid tissue; N/A, not applicable; PBMC, peripheral blood mononuclear cell.

Table 3. Detailed overview of immune function markers

	Parameter (immune function marker)	Type of assay/specific method	Major advantages	Major disadvantages	Biological functions	Examples of clinical significance	Confounding variables (exercise, age)	Comments
1. <i>In vivo</i> integrated responses	Response to vaccination	Vaccine-specific antibodies in serum/plasma	Integrated, <i>in vivo</i> measure	No repeated measurements possible	Primary B cell responses (<i>Pneumococcus</i>)	Reflects <i>in vivo</i> immune function and vaccination status	No effect with acute exercise or training (though may be higher in fit <i>v.</i> sedentary elderly)	History of vaccinations should be assessed
		Vaccine-specific T cell response	In plasma and/or <i>ex vivo</i> stimulation of lymphocytes	No cross-over design studies with the same vaccination	Primary T and B cell responses (first exposure to vaccine)		No general decline with age, but response declines faster in elderly (less for <i>Pneumococcus</i>)	Used widely
		Vaccine-specific antibody forming cells	Primary and secondary (boosting) responses Dynamic response		Memory T and B cell response (booster vaccine)			Boosting effect <i>in vivo</i>
2. <i>Ex vivo</i> immune cell functions	Delayed-type hypersensitivity	Mantoux test (CMI Multitest® kit)	Integrated, <i>in vivo</i> measure	Semi-quantitative History of vaccination interferes Boosting possible with repeated applications	<i>In vivo</i> cell-mediated immune response	Significant inverse correlation with mortality in aged, critically ill and post-surgery patients	↓ with acute exercise No effect of training ↓ with age	Semi-quantitative
	Innate immune functions	Phagocytosis	Possibility to simultaneously measure phagocytosis, oxidative burst and killing	Important variations with clinical status (in number of cells and functions)	Measure of neutrophil and monocyte function(s)	Related to ability to combat bacterial infections	= or ↑ with acute exercise ↓ with training = or ↑ with ageing	
		Oxidative burst	See above	See above	See above	Related to ability to combat bacterial infection	↓ with acute exercise ↓ with training ↓ with ageing	
	NK cell function	NK cell-mediated cytotoxicity	Particularly sensitive to diet and stress	Need for K562 target cell line	Non MHC-related cytotoxic function	Low activity is correlated with increased cancer risk	= or ↓ function per cell but ↑ numbers during acute exercise ↓ function and numbers after 2–48 h ↓ per cell, but ↑ cell number with ageing	
		⁵¹ Cr-release from K562 cell line		Radioactive technique (⁵¹ Cr)	Reflects spontaneous defense against virally infected and malignant cells			

Table 3. Continued

Parameter (immune function marker)	Type of assay/specific method	Major advantages	Major disadvantages	Biological functions	Examples of clinical significance	Confounding variables (exercise, age)	Comments
APC function (usually limited to blood monocytes)	Cell culture; then: ELISA RT-PCR Flow cytometry	Possible investigation of monocytes separately, cytokine production linked with monocyte sub-population	Inflammatory response (TNF, IL-1) Anti-inflammatory response (IL-6, IL-1ra, IL-10)	Measure of cell activation and capability of cells to produce cytokine profile Inflammatory mediators	TNF, IL-1 ↑ with acute exercise Conflicting data with ageing IL-6 production per cell ↓ with acute exercise	Kinetics different in old and young subjects IL-6 correlates with CRP	
Intracellular cytokine accumulation	Cell culture and treatment; then: Flow cytometry	Allows identification of cell-specific cytokine production		Measure of cell activation MHC class II expression related to antigen-presenting capacity Measure of cell activation	↑ PGE ₂ with exercise ↑ PGE ₂ with aging ↓ with acute exercise	Related to type of fatty acids (n-6:n-3) in diet	
Eicosanoid (PGE ₂) production Activation marker (CD80/86) and MHC class II expression	Cell culture; then: ELISA GC/MS, HPLC/MS Cell culture; then: Flow cytometry			Measure of cell activation MHC class II expression related to antigen-presenting capacity Measure of cell activation	↓ with acute exercise		
TLR expression	Cell culture; then: Flow cytometry		Labelled antibodies not available for all TLR	Measure of cell activation			
Acquired immune functions	Cell culture; then: ³ H-labelling of DNA Flow cytometry ELISA (BrdU)	Reflects overall responsiveness of T cells Use of cell-specific mitogens or stimulants (PHA, PWM, Con A, IL-2, anti-CD3) Can distinguish early and later stages of activation May be done after cell fixation (24 h delay)	Variable results due to different stimulants	Measure of lymphocyte replication	PHA response correlates inversely with mortality in HIV (PWM) and ageing (PHA, PWM)	↓ with acute exercise ↓ with training ↓ with age	
Lymphocyte activation	Cell culture; then: Flow cytometry		Measure only sub-populations of cells, not a true functional marker	Quantification of activated cells	Inflammatory process, changes only with major activation CD69 ↓ with acute exercise HLA-DR ⁺ , CD95 ⁺ , CD28 ⁻ ↑ with age (entire life)		

Table 3. Continued

Parameter (immune function marker)	Type of assay/specific method	Major advantages	Major disadvantages	Biological functions	Examples of clinical significance	Confounding variables (exercise, age)	Comments
Lymphocyte-derived mediator production	Cell culture; then: ELISA RT-PCR Flow cytometry	Possible to investigate lymphocytes separately, cytokine production linked with lymphocyte sub-populations		TH1 response (IL-2, IFN- γ) TH2 response (IL-4, IL-5)	Increased IL-4, IL-5 in aged persons Low IL-2 production increases infectious diseases in aged persons	\downarrow per cell with acute exercise (IL-2, IFN- γ) No change per cell with acute exercise (IL-4) \uparrow or = with age (IL-2) \uparrow with age (IL-4, IL-5)	Best <i>ex vivo</i> indicator of cell-mediated immunity
Intracellular cytokine accumulation	Cell culture; then: Flow cytometry	Allows identification of lymphocyte sub-populations producing specific cytokines		Measure of <i>in vivo</i> cell activation Capability of cells to produce cytokine profile Opsonisation Bacterial lysis		TH1:TH2 ratio \downarrow with exercise and ageing	
Complement activity	ELISA; then: Lysis of sheep red blood cells	Sensitive indicator of protein deficiency	Apparently insensitive to other dietary changes				
Circulating levels of Ig (IgG, IgM, IgA)	ELISA	Normal ranges exist	Not antigen-specific, sensitive	Detection of important B cell defects or unspecific polyclonal activation		IgG, IgA \uparrow with age and chronic infections \sim 5–15% \uparrow with acute exercise due to haemoconcentration	Do not change with treatment Limited use
Differential leucocyte count	Automated differential cell count	Routinely available Normal ranges exist		Indicates circulating leucocyte pool	Redistribution after stressors		
Lymphocyte sub-populations	Flow cytometry, combination of 3 to 4 markers		Must run a panel of markers	Marker of current and previous activation, increases over life span	CD45RO increase with age/exercise	CD45RO \uparrow and CD45RA \uparrow with acute exercise but \uparrow CD45RO:CD45RA ratio	
Ratio of memory to native cells (CD45RO:C-D45RA) lymphocytes							CD45RO \downarrow and \downarrow CD45RO:CD45RA ratio with training CD45RO \uparrow , CD45RA \downarrow with age

Table 3. Continued

Parameter (immune function marker)	Type of assay/specific method	Major advantages	Major disadvantages	Biological functions	Examples of clinical significance	Confounding variables (exercise, age)	Comments
Other sub-population analysis: whole blood phenotypes	Flow cytometry, combination of several markers		Must run a panel of markers		Low CD4 percentage predicts mortality in elderly	CD4 ↓ with age CD4 ↓ with undernutrition	
Circulating cytokine and cytokine receptor concentrations	High-sensitivity ELISA	Reflects <i>in vivo</i> activation (IL-1, TNF, IL-6 and IL-1ra can be quantified with high sensitivity)	Highly sensitive antibodies not available for some cytokines including IL-2 and IL-4	Reflect <i>in vivo</i> pro- and anti-inflammatory state		CD8 ↓ with ageing IL-6, IL-1ra, TNF ↑ with acute exercise TNF, IL-6, IL-1, sTNFR ↑ with ageing	Plasma levels reflect production by many tissues (30% of plasma TNF-α from adipose tissue)
Other	High-sensitivity ELISA	Reflects <i>in vivo</i> activation	Difficult to extend interpretation to larger molecules	IL-10 reflects anti-inflammation IL-2 receptor reflects T cell activation		IL-10 ↑ with acute exercise	
4. Gut-associated immune functions	Urinary appearance of sugars	No venepuncture required		Barrier to invasion of pathogens	Defence against microbial and antigen invasion		
Bacterial permeability Serum endotoxins	16S ribosomal DNA translocation ELISA	No endoscopy required Good assessment of bacterial transfer	Analysis requires chromatography Venepuncture Relatively expensive molecular analysis		Barrier function	Inflammation = or ↑ serum endotoxins with acute prolonged exercise	
GALT plasma secretory cell function	ELISA	Non-invasive	Information about cellular immune system restricted to B cell/plasma cells	Neutralisation of luminal antigens	GALT B cell activity	Changes in childhood	IgA increase in childhood
Faecal water IgA	No endoscopy required	No endoscopy required				sigA =, ↑ or ↓ with acute exercise sigA ↑ with acute psychological stress sigA ↓ with heavy training and chronic psychological stress	

Table 3. *Continued*

Parameter (immune function marker)	Type of assay/specific method	Major advantages	Major disadvantages	Biological functions	Examples of clinical significance	Confounding variables (exercise, age)	Comments
Intestinal inflammation	Stool calprotectin	Stool sample only	No information about normal immune system in young, old or sportspeople		Defence molecule		
Assessment of mucosal histology and Peyer's patches	Endoscopy and biopsy	Detailed information about T cell function and mucosal architecture	Degree of invasiveness prevents it being used in nutritional assessment of immune function				
Stool cytokine concentration	ELISA	Information about mucosal activation	Intestinal cytokine production				Activity reflects <i>in vivo</i> response
5. Questionnaires of infections	Standardised questionnaires	Allows validation of immunological measurements	Not reliable except if performed in a standardised way				↑ 1–2 weeks after acute prolonged competitive exercise events
Follow up of patients, e.g. 'days off from work', 'days with use of antibiotics'	Standardised questionnaires	Allows validation of immunological measurements	Not reliable except if performed in a standardised way				

NK, natural killer; APC, antigen-presenting cell; GALT, gut-associated lymphoid tissue; PBMC, peripheral blood mononuclear cell; PGE, prostaglandin E; TLR, toll-like receptor; HLA, human leucocyte antigen; BrdU, 5-bromo-2-deoxyuridine; PHA, phytohaemagglutinin; PWM, pokeweed mitogen; Con A, concanavalin A; ra, receptor antagonist; TH, T helper; IFN, interferon; ↓, decreased; ↑, increased; sTNFR, soluble TNF receptor; sIgA, secretory IgA; CRP, C-reactive protein.

effect of nutrients on protective *in vivo* immune responsiveness (Meydani *et al.* 1997; Van Loveren *et al.* 1999, 2001).

Delayed-type hypersensitivity response

DTH responses are local cell-mediated responses that are triggered only in sensitised individuals by the intracutaneous administration of antigen. The DTH response can be measured as epidermal induration 24–48 h after antigen application, which reflects the integrated outcome of a cell-mediated immune response (Ananworanich & Shearer, 2002). The prototype DTH response is the Mantoux test to diagnose exposure to *Mycobacterium tuberculosis*. The CMI Multitest[®] was developed to simultaneously administer seven different common antigenic preparations. The dose levels of these preparations were minimised to prevent induction of immunological memory, provided that the test was not used too many times. The test could therefore be used to assess modulation of cellular immune responsiveness by comparing reactions before and after nutritional intervention (Lesourd *et al.* 1985). As such, the test has been used in numerous nutrition studies but, unfortunately, the CMI Multitest[®] kit is no longer commercially available. In addition, the individual outcome of the CMI Multitest[®] was highly variable depending on the subject's vaccination history.

Application of antigenic material by syringe or prick similar to the Mantoux test could be considered as an alternative but since application only yields DTH responses in sensitised subjects, it is important to apply a range of antigens. Sleijffers *et al.* (2001) measured DTH responses to an uncommon contact sensitiser (diphenylcyclopropenone) after cutaneous sensitisation. Although the deliberate sensitisation may raise some concern, this could potentially be an attractive option as it offers control over both the sensitisation and the elicitation phase of the DTH response. A promising new approach to measure DTH first requires vaccination (e.g. against hepatitis B), which is followed by an intradermal application of the same antigen. However, data from human studies using this approach are not yet available.

DTH responses decline with age (Marrie *et al.* 1988; Fietta *et al.* 1994) and are inversely correlated with mortality in surgery patients (Bradley *et al.* 1981; MacLean, 1988; Christou *et al.* 1995) and cancer patients (Aziz *et al.* 1998), with progression to AIDS in persons with HIV infection (Blatt *et al.* 1993; Gordin *et al.* 1994), with the risk of URTI (Zaman *et al.* 1997) and with progression of acute to persistent diarrhoea in Bangladeshi children (Azim *et al.* 2000). DTH responses that rely on the uncontrolled history of exposure to the antigen have an inherently large inter-individual variation and are therefore not well suited to compare immune responsiveness of individuals or small groups based on a single application. Instead, changes within an individual should be assessed by comparing multiple DTH tests on that individual. It is also essential to standardise the semi-quantitative evaluation of DTH responses. After a strong DTH response, subjects may experience prolonged discoloration/irritation at the site of the response. Despite these limitations, DTH responses are sensitive *in vivo* indicators of the ability to mount cell-mediated immune responses that have been used successfully in nutrition immunology studies, particularly in subjects with compromised immune function, e.g. due to exposure to uv light (Fuller *et al.* 1992; Herraiz *et al.* 1998), and in elderly subjects (Meydani *et al.* 1990; Bogden *et al.* 1994; Pallast *et al.* 1999).

Ex vivo immune functions

Innate immune system

Phagocyte activity. Phagocytes have an important role in the engulfing and killing of extracellular pathogens and in the removal of antigen–antibody complexes. Neutrophils are the main phagocytic cells in the blood, but monocytes also have some phagocytic activity. Traditionally, phagocytosis was assessed microscopically by counting ingested particles such as erythrocytes, bacteria or latex spheres. The most effective method to measure phagocytosis is based on a flow cytometer, which, today, is standard equipment in many laboratories. With a flow cytometer, the internalisation of fluorescently labelled particles or cells can be measured very efficiently; at the same time this method effectively differentiates between membrane-bound and internalised particles or cells (Lehmann *et al.* 2000; O'Gorman, 2002). The method provides information on the number of neutrophils and monocytes involved in phagocytosis (percentage of cells that have internalised particles or cells), as well as the level of activity (quantity of internalised particles per active cell expressed as mean or median fluorescence intensity). Flow cytometry also enables the assessment of oxidative burst (the percentage of cells producing reactive oxygen molecules and the mean or median fluorescence intensity per cell) that is triggered by the phagocytosis of bacteria and serves to kill them. Provided that the assay conditions are rigorously standardised (concentration of particles or labelled cells, timing, temperature, selection of stimulus), the assay has a relatively low within- and between-subject variation. A further advantage of the flow cytometric method is that both phagocytosis and oxidative burst can be measured at the same time.

Another aspect of neutrophil function that can be measured *in vitro* is the degranulation response to bacterial LPS (Robson *et al.* 1999). Whole blood is incubated with LPS, followed by determination of the amount of elastase released using an ELISA kit specific for polymorphonuclear cell elastase. This method correlates with measures of oxidative burst activity and therefore can be an alternative method for evaluating neutrophil function for laboratories that do not have access to a flow cytometer. These functions demonstrate important variations with clinical status and play an important role in the first defence to bacteria and fungi (Kuritzkes, 2000; Lord *et al.* 2001; Fidel, 2002).

Natural killer cell activity. NK cells are large, non-T, non-B lymphocytes with an important role in the defence against viruses and other intracellular pathogens. They kill infected and transformed target cells and in the presence of IL-2 contribute to lymphokine-activated killing (lymphokine-activated killer cell activity). To measure NK cell activity, NK-sensitive K562 target cells are briefly co-cultured with NK cells (in peripheral blood mononuclear cells, PBMC) at different ratios. The target cells are frequently pre-labelled with ⁵¹Cr or a fluorescent dye (containing non-radioactive Eu³⁺). Subsequent target cell lysis can be measured by the release of radioactivity or fluorescent dye (Nagao *et al.* 1996), or, alternatively, by the release of cytoplasmic enzymes such as lactate dehydrogenase (Decker & Lohmann-Matthes, 1988; Konjevic *et al.* 1997) or by flow cytometric assessment of the uptake of a DNA stain by fluorescently labelled target cells (Provinciali *et al.* 1992; Chang *et al.* 1993). The traditional ⁵¹Cr method has the disadvantage of using a radioisotope with a short half-life, potential environmental and health hazards, and the spontaneous release of ⁵¹Cr from labelled target cells,

which increases inter-assay variability. However, when highly standardised protocols are used, the results obtained using flow cytometric analysis of target cell DNA staining are identical to those of the ^{51}Cr method (r 0.91; Chang *et al.* 1993). All methods require a source of the target cells (K562) for the NK cell lytic activity assay. Target cells should be controlled for mycoplasma on a regular basis to avoid variable sensitivity of the targets to NK cells (Whiteside *et al.* 1990). NK cell activity can also be measured in cryopreserved NK cells (Fujiwara *et al.* 1986). However, in some individuals cryopreservation, even under optimal conditions, can decrease NK cell activity (Whiteside *et al.* 1990). Therefore, the use of fresh NK cells is recommended.

Activity of NK cells appears to be among the immune functions most sensitive to dietary modulation. This may be due to the fact that NK cells are highly dependent on cytokines and are constitutively activated (i.e. they kill unless signalled not to). NK cell activity indicates the spontaneous defence against virus-infected and malignant cells. Low NK cell activity is correlated with increased cancer risk (Imai *et al.* 2000) and with increased mortality in the elderly (Ogata *et al.* 2001).

Monocyte-derived mediators. During an infection, various cell types produce peptide (cytokines) and lipid (eicosanoids) mediators with pro- or anti-inflammatory activity. Typically, production of such mediators is assessed in supernatants of PBMC or whole blood cultures after stimulation of the monocytes with Gram-negative bacteria or LPS thereof. These mediators can subsequently be measured in various ways. ELISA are the most frequently used for this purpose. Alternatively, the number of cells producing a particular cytokine can be enumerated using enzyme-linked immunospot assays or flow cytometric analyses of intracellularly labelled cytokines. The latter has the advantage that the phenotype of the producing cells can be determined. Ideally, a cytokine production profile should be determined by examining both pro- (TNF- α , IL-1 β) and anti- (IL-1ra, IL-10) inflammatory factors. In comparison with extracellular cytokine detection, intracellular cytokine quantification requires an even more standardised protocol due to the need for additional reagents such as membrane permeabilisers and Golgi apparatus blockers. The most sensitive method to quantify cytokine mRNA levels is reverse transcription in combination with PCR (RT-PCR). To achieve a high reproducibility of quantitative analysis, it is recommended to use a highly standardised protocol with reagents all purchased from the same vendor (Kruse & Rieckmann, 2002).

For all antibody-based assays (ELISA, flow cytometry), the quality of the antibodies selected for capture and detection of a cytokine is the most crucial factor for specificity and sensitivity of the assay (Remick, 2002). Using isolated monocytes allows the amount of the produced cytokine to be related to a defined cell number. In contrast, the whole blood culture technique measures cytokine production by a known volume of blood. However, this approach does not control for differences in blood cell numbers. To assess cytokine concentrations at different time points (pre- and post-intervention) in longitudinal studies, it is practical to use serially collected frozen aliquots of culture supernatants for cytokine measurements within the same ELISA plate. This will strongly decrease inter-assay variability. The level of cytokine production appears to be characteristic of an individual, resulting in low within-subject variability (5–10%; Bienvenu *et al.* 2000). In contrast, marked between-subject variation has been reported with up to sixteen-fold variation in cytokine production by mononuclear cells, which seems likely to be related

to polymorphisms in the genes that control cytokine production (Yaqoob *et al.* 1999). It has to be kept in mind that the presence of soluble cytokine receptors in biological samples can affect the recognition of cytokines by immunoassays.

It has been suggested that production of eicosanoids, of which PGE₂ has been measured most frequently, may be related to the type of PUFA (n -6: n -3) in the diet. However, the physiological relevance of this measurement remains to be determined.

Antigen-presenting cell function. Monocytes, macrophages and dendritic cells act as APC and so, in addition to their role as phagocytes, are important in initiating and regulating the adaptive immune response. TLR on macrophages recognise conserved molecular patterns on microbes and initiate intracellular signalling pathways that result in up-regulation of co-stimulatory molecule (CD80/86) and MHC class II protein expression and cytokine production. Expression of TLR (e.g. TLR1, 2, 4 and 9) on CD14⁺ monocytes can be quantified by flow cytometry and the extent of APC activation can be assessed by quantifying CD80, CD86, HLA-DR and MHC class II expression or cytokine (e.g. IL-6) production following stimulation with ligands specific for different TLR. The specific pathogen-associated molecular patterns recognised by TLR family members have been well characterised: TLR2 homodimers and TLR2–TLR1 and TLR2–TLR6 heterodimers mediate responses to lipoproteins, peptidoglycan, lipoteichoic acid and zymosan; TLR3 to double-stranded RNA; TLR4 to LPS; TLR5 to bacterial flagellin; TLR7 and 8 to imidazoquinolines; TLR9 to bacterial DNA. At present, the clinical significance of these measures of APC function is unclear but it is known that TLR expression and function are influenced by exercise stress and ageing (Renshaw *et al.* 2002; Lancaster *et al.* 2003c). *In vitro* stimulation of monocyte MHC class II protein expression (HLA-DR, -DP, -DQ, etc.) is a useful marker of APC function. Failure of *in vivo* HLA-DR expression in the monocytes of critically ill patients is a poor prognostic factor for survival. DR is the HLA (human leucocyte antigen) type that has most known disease associations and expression of the other HLA is largely co-regulated. *In vitro* stimulants include cytokines (e.g. IFN- γ) and bacteria.

Adaptive immune system

Lymphocyte proliferation. Lymphocytes normally exist as resting cells. Upon stimulation they either enter a pathway of activation-induced cell death, or they become activated and proliferate to expand the number of antigen-specific lymphocytes before these differentiate into effector and memory cells. Activation-induced cell death can be measured using robust, validated techniques such as annexin V assays that are commercially available and flow cytometry. However, this is not frequently measured in nutrition studies and it is not clear how it relates to resistance to infections. Activation and proliferation of lymphocytes, on the other hand, are often used as indicators of lymphocyte reactivity. Typical stimuli to activate lymphocytes are pokeweed mitogen that stimulates all lymphocytes and the plant lectins phytohaemagglutinin and concanavalin A that stimulate all T cells. T cells can also be activated with combinations of certain antibodies (i.e. anti-CD3 combined with anti-CD28). The latter resembles the physiological activation more closely and may therefore be preferred (Nisbet-Brown *et al.* 1987; Meyaard *et al.* 1995). If possible, it is recommended to combine one or

more of these polyclonal stimuli with a more specific stimulation, for instance by using the antigen from a vaccine.

For *ex vivo* stimulation of lymphocytes, usually PBMC are isolated and cultured in medium containing exogenous serum, often fetal bovine serum. However and particularly in nutritional studies, it is important to realise that during isolation and culture the cells' environment, i.e. subset distribution, cell-to-cell contact, exposure to hormones and, most importantly, available nutrients, are changed in such fetal bovine serum-supplemented culture systems. 'Whole blood cultures' provide a pragmatic alternative for the artificial and labour-intensive PBMC cultures. In whole blood assays heparinised blood is cultured undiluted or diluted (1:5 or 1:10) in medium without exogenous serum in the presence of antigen or mitogen (Bloemena *et al.* 1989; Elsasser-Beile *et al.* 1991). Pre-assay isolation and counting of cells are not required and cells are cultured in their naturally occurring ratios and in autologous plasma. If desired, production of cytokines per fixed number of particular cells can be calculated after completion of the assay. It is difficult, however, to determine which cells produce which cytokines and effects of nutrients on one cell type can lead to indirect effects on others. Isolated PBMC cultures may therefore be preferred for mechanistic studies, in which case autologous serum or, if that is not possible, pooled human serum or serum-free medium could be considered as an alternative to fetal bovine serum. If fetal bovine serum is used, it is important to use the same batch for all experiments. Proliferation is typically measured as [³H]thymidine incorporation into the DNA of proliferating cells, which requires radioisotope handling and access to a scintillation counter. However, alternative methods have been developed assessing incorporation of the thymidine analogue bromodeoxyuridine using ELISA (Martinon *et al.* 1987) or flow cytometry (Gaines *et al.* 1996), or fluorescent staining of DNA using propidium iodide. Whereas the sensitivity of the ELISA method is lower than that of [³H]thymidine incorporation, its within-subject variability is low (B Watzl, unpublished results). Flow cytometric analysis can be combined with a phenotypic analysis of the proliferating cells and the correlation between this method and [³H]thymidine incorporation is very high (r 0.92; Messele *et al.* 2000). Proliferation has been measured using cryopreserved human PBMC (Allsopp *et al.* 1998).

An emerging method is the staining of membranes with a fluorescent dye like carboxyfluorescein diacetate succinimidyl ester that dilutes with each round of division (Lyons, 2000). This method can easily be combined with staining of surface markers to distinguish relevant subsets (i.e. CD4, CD8, CD45RO, CD45RA). Although the method requires further standardisation, it appears to be more robust and less variable than [³H]thymidine incorporation and has the advantage that one sample can provide detailed information on the nature of the dividing cells and the number of divisions. Although lymphocyte proliferation has been measured for decades, the methodology is not standardised between laboratories and surprisingly little information is published on variability within individuals and reference ranges. To allow proper interpretation of differences and changes observed, it is therefore important to measure and report the variability within subjects during a study.

Lymphocyte proliferation is one of the most frequently used immune function markers and provides a relatively simple means to determine the ability of lymphocytes to replicate after stimulation. However, results in nutrition studies have been

variable, which may, in part, be due to the use of different stimuli that act at different stages of the activation cascade. Lymphocyte proliferation has been shown to correlate inversely with mortality in HIV patients and in elderly subjects (Murasko *et al.* 1987; Hofmann *et al.* 1989; Schellekens *et al.* 1990).

Lymphocyte activation. The activation of lymphocytes can also be assessed by their surface expression of activation markers such as CD69, CD95 and HLA-DR. The use of CD25 as an activation marker has become more complicated since it has been identified as a marker on regulatory T cells. Expression of activation markers is measured by flow cytometry and can easily be combined with further phenotypic analysis of the activated cells, which can be done after a delay of up to 24 h provided that the cells are fixed after staining. By using antibodies to different activation markers, it is possible to distinguish early (CD69) and late stages (HLA-DR and CD95) of lymphocyte activation. Spontaneous expression of activation markers can be determined on lymphocytes in freshly isolated blood. This is indicative of the *in vivo* activation of various lymphocyte subsets. Expression can also be assessed after *ex vivo* stimulation with the same stimuli used to induce lymphocyte proliferation but mitogen-induced expression of CD69 by T cells is less sensitive as an indicator of proliferation than [³H]thymidine incorporation (Hutchinson *et al.* 1999). However, it does appear to be more robust and allows determination of the phenotype of the activated cells.

Lymphocyte-derived mediators. Although it is relevant to know the ability of lymphocytes to become activated and to proliferate, this reveals little about their functional capabilities. For this, functional assays are essential. The function of TH cells is typically assessed as production of cytokines. TH1 and TH2 lymphocytes can be distinguished by the type of cytokines they produce, whereas the level of production indicates the level of activity. TH1 cells typically produce IL-2 and IFN- γ , TH2 cells IL-4, IL-5 and IL-13, and regulatory T cells, IL-10 and TGF- β . To functionally characterise T cells, lymphocytes are often stimulated in culture and the concentrations of various cytokines in the supernatants are assessed using ELISA. Alternatively, mRNA for cytokines can be assessed using RT-PCR, or the number of lymphocytes that produce a particular cytokine can be enumerated using enzyme-linked immunospot or flow cytometry (Vignali, 2000; Kruse & Rieckmann, 2002; Remick, 2002). Again, flow cytometric analysis has the advantage that it can be combined with phenotypic analysis of the producing cells. For this, lymphocytes need to be activated in the presence of a protein transport disrupter such as Brefeldin A, which blocks cytokine secretion resulting in intracellular accumulation of cytokines. Ideally, a cluster of cytokines should be assessed to cover TH1, TH2 and regulatory T cells functions (i.e. IFN- γ , IL-2, TGF- β , IL-10, IL-5 and IL-4). As such, production of lymphocyte-derived cytokines following activation provides mechanistic information regarding the immunomodulatory activity of TH cells.

Activity of cytotoxic T cells is typically characterised by lysis of antigen-loaded MHC-matched target cells. As human subjects vary widely in their MHC haplotype, this is in practice very difficult to measure and has rarely been used in nutrition studies in man. Production and release of perforin and granzymes assessed by RT-PCR or intracellular staining have been used as surrogate for T cell (and NK cell) cytotoxicity but data on robustness, variability and correlation with relevant clinical endpoints are scarce.

Antibody production. The function of B lymphocytes can be assessed as *ex vivo* production of antibodies. Traditionally, this

was done using plaque-forming cell assays, which turned out to be among the markers most predictive of resistance to induced infections in mice using multivariate statistical analyses of immunotoxicological data (Keil *et al.* 2001). Nowadays, antibody production is typically assessed with an ELISA to determine the concentration of antibodies in the culture supernatant or with an enzyme-linked immunospot assay to enumerate the number of B cells producing antibodies. With both methods detection can be tailored to total (sub) class of antibodies (IgM, IgG, etc.), to antibodies of a particular isotype (IgG1, IgG4, etc.) or to antibodies with particular antigen specificity. These antibodies can be measured without stimulation to assess the spontaneous/*in vivo*-induced production, or their production can be induced *ex vivo* by culture in the presence of a polyclonal stimulus like pokeweed mitogen or LPS, or with a specific antigen. The latter appears to be most informative, particularly if used to assess the kinetics of vaccine-specific antibody production following vaccination.

Basal markers of immune functions

Complement activity

Complement consists of a series of plasma pro-enzymes synthesised in the liver with a pivotal role in the elimination of pathogens. Complement can be activated by micro-organisms and antibody–antigen complexes. When triggered, this enzyme cascade results in lysis of micro-organisms and enhanced phagocytosis due to opsonisation. Complement factors such as C3 have been used as indicators of decreased protein synthesis during PEM, but have not generally been found responsive to other dietary changes in non-malnourished subjects. The concentrations of complement factors such as C3 and C4 are typically assessed using ELISA and give an indication of complement reserve capacity. The concentrations of activated complement fragments such as C3a and C5a *in vivo* can be used as a very good indicator of complement activation and inflammation. This requires the immediate separation of EDTA plasma using EDTA as anticoagulant, but the plasma can be frozen so that assay of activation fragments can be done later. Complement function can be assessed by titrating the lysis of sheep red blood cells. This requires the immediate separation of serum, but this can be frozen so that assay measurements can be done later.

Circulating levels of immunoglobulins

Serum levels of Ig have been used to assess immune status. Typical ranges in adults for these are: IgA, 1.4–4.0 mg/ml; IgD, 0–4 mg/ml; IgE, 17–450 ng/ml; IgG, 8–16 mg/ml; IgM, 0.5–2.0 mg/ml (Cummings *et al.* 2004). Conditions with impaired protein synthesis such as PEM result in diminished Ig levels. However, in less extreme cases total levels of Ig are not very responsive to dietary changes and slight variations cannot be interpreted clinically and are of limited use in nutrition studies. However, if the immune system is specifically challenged by a vaccination, detection of antigen-specific antibodies in the serum is the easiest way to assess the adaptive immune response to the antigen. Discrimination of antigen-specific Ig of different isotypes in serum and saliva may provide additional information on the type of immune response elicited (IgG1 and IgG3 indicating TH1-driven responses and IgG4 and IgE indicating

TH2-driven responses). During chronic infections, serum levels of IgG and IgA tend to increase. Secretory IgA concentration can be used as an indicator of mucosal immunity (discussed on p. 471).

Circulating acute-phase protein, cytokine and cytokine receptor concentrations

In the absence of a controlled stimulation such as a vaccination, deviant levels of acute-phase proteins such as CRP, α -glycoprotein, albumin and prealbumin in serum or plasma are indicators of ongoing (sub-) clinical infections or other diseases. As such these can be used to help interpret the results of functional assays but by themselves they cannot be extrapolated to immune function or resistance to infections. Using highly sensitive ELISA, various cytokines and soluble variants of cytokine receptors and adhesion molecules involved in leucocyte–endothelial cell interactions can also be measured in serum (or plasma). However, variations have been reported between ELISA kits from different suppliers, partially explaining the wide differences between laboratories. Comparative studies indicate that no difference exists between levels of individual cytokines, apart from IFN- γ and IL-10, in plasma and serum (Remick, 2002). Recently, Komatsu *et al.* (2001) described a highly sensitive method for measuring cytokines directly from serum. With immuno-PCR assay, limits of detection were 0.001 ng/l for TNF- α compared with 50 ng/l in an ELISA. This new assay will allow scientists to collect more accurate data on cytokine concentrations in blood. Cytokines, cytokine receptors and soluble adhesion molecules are released during ongoing (inflammatory) immune responses and are affected by certain diseases. Some of these factors can therefore be used as biomarkers for development of diseases that involve early endothelial activation and inflammation. For instance, increased plasma levels of the soluble form of intercellular adhesion molecule-1 correlate with increased risk for development of CVD. However, the relevance of the circulating forms of cytokines, cytokine receptors and adhesion molecules to immune responsiveness of healthy individuals in the absence of a controlled *in vivo* stimulus is currently unclear. It is important to realise that the concentrations found in the blood are the net outcome of production in various tissues, including muscle and fat tissue, and degradation by various cells and tissues.

Leucocyte and lymphocyte subsets

The various cell populations in the blood can be determined by routine haematology, often followed by flow cytometric analysis of lymphocyte subsets. This can provide useful information, for example on redistribution of the leucocyte pool triggered by various stressors including fasting and refeeding (Walrand *et al.* 2001). However, by themselves, absolute or relative amounts of different leucocyte types are physiologically relevant only in extreme cases such as severe malnutrition and AIDS. On the other hand, haematological and phenotypic analyses do define the cell populations that are used in functional assays and as such provide essential background data for the interpretation of those assays. Standardised systems are nowadays available for the semi-automated multi-parameter quantification of the relative, and after addition of an internal standard, also of the absolute numbers of the main lymphocyte subsets (Table 4). These will

Table 4. Normal ranges for immune cell numbers in the circulation of human adults

	Number of cells ($\times 10^9$) per litre blood*	% of total leucocytes
Total leucocytes	4.0–11.0	100
Neutrophils	2.0–7.5	50–80
Eosinophils	0–0.4	0–5
Basophils	0–0.1	0–2
Monocytes	0.2–0.8	2–10
Lymphocytes	1.0–3.5	25–50
T lymphocytes	0.6–2.5	
Helper T lymphocytes (CD3 ⁺ CD4 ⁺)	0.35–1.5	
Cytotoxic T lymphocytes (CD3 ⁺ CD8 ⁺)	0.23–1.1	
B lymphocytes (CD19 ⁺)	0.04–0.7	
Natural killer cells (CD3 ⁻ CD56 ⁺)	0.2–0.7	

*5th and 95th percentile (MacLennan & Drayson, 1999).

typically include TH cells (CD4⁺), cytotoxic T cells (CD8⁺), B cells (CD19⁺) and NK cells (CD16⁺CD56⁺), but can be extended, for instance, to distinguish naïve (CD45RA⁺) and memory cells (CD45RO⁺).

Gut-associated immune functions

The mucosal immune system is arguably the largest immune component in the body. It defends not only the intestine to invasion by infections, but also plays a similar role in the respiratory system, mouth, eyes and reproductive tract. However, it is that of the intestine that is most widely characterised.

The immune system of the gut divides into the physical barrier of the intestine and the active immune components, which include both innate and adaptive immune responses. The physical barrier is central to the protection of the body to infections. Acid in the stomach, active peristalsis, mucus secretion and the tightly connected monolayer of the epithelium each play a major role in preventing microbial organisms from entering the body.

The cells of the immune system are organised in a complex pattern within the intestine (Fig. 1). Specialised lymphoid tissue with germinal centres, the Peyer's patches, reside below specialised epithelia. This lymphoid-associated epithelium includes M cells, whose structure enables sampling of small particles. Furthermore, other lymphocytes are present both in the lamina propria and associated with the epithelium itself. All cells of the immune system are found in the lamina propria (Fig. 1).

The intestinal epithelium is topologically on the surface of the intestine. However, because of the inversion of the intestine into a tube within the body, its surface is very inaccessible to observers of its function. Currently, testing the immune system of the intestine is achieved only indirectly without very invasive techniques. The latter include endoscopy and biopsy.

Strategies for testing the immune system in the intestine are currently very modest when compared with those used to test systemic immune function. Tests designed to examine the integrity of the intestinal barrier include: (i) those in which markers are given orally (most often a non-metabolisable sugar) and then measured in the urine; (ii) the passage of resident bacteria from the lumen of the intestine into the circulation.

A direct examination of the ability of the intestine to resist infection induced by attenuated pathogens is a promising approach. Bovee-Oudenhoven *et al.* (2003) administered a live but attenuated enterotoxigenic *E. coli* strain (strain E1392/75-2A) to human volunteers in a study of the efficacy of Ca in

enhancing the mucosal immune system and measured clinical response thereafter. Such an approach requires careful attention to the virulence of the infectious agent used and specific ethical considerations.

The most useful marker of the mucosal immune system is IgA. However, measuring elements of the adaptive response relies on the assumption that secretion of Ig, for example, in the mouth reflects that of the rest of the mucosal immune system.

Immunoglobulin A

IgA is the predominant Ig secreted at mucosal surfaces. IgA is a dimer of 350 kDa. The two monomers are joined by a J chain and protected from proteolysis by another peptide, the secretory component, made by epithelial cells. It is acquired by IgA molecules as they pass through the epithelium on their journey from the plasma cell to the mucosal surface. IgA can immobilise microorganisms or prevent their attachment to mucosal surfaces. Circulating IgA is mostly monomeric. It is generally believed that most IgA in the blood is later available for transport to mucosal surfaces.

The production of IgA at birth is very small and it increases slowly. A 2-year-old child has a serum concentration of IgA that is half that of adults. IgA levels at mucosal surfaces increase in a similar way.

Salivary IgA can be measured by an ELISA method with a CV of 5–10%. There is some evidence that low levels of salivary IgA are associated with increased incidence of URTI (Isaacs *et al.* 1984; Mackinnon, 1999; Gleeson, 2000). For the assessment of IgA, unstimulated saliva collections should be made at rest in the post-absorptive or fasted state (Gleeson, 2000) and at specific times during the day and circadian rhythms, because changes in salivary IgA also occur with acute changes in salivary flow rate. When flow rate increases due to stimulation (e.g. chewing), then salivary IgA concentration falls. Conversely, if salivary flow rate falls (e.g. in dehydration or heavy exercise), then the salivary IgA concentration increases. The concentration of IgA in saliva is decreased during periods of chronic physical or psychological stress.

Finally, various markers for measuring inflammation in the intestine are available for measuring neutrophil components in the stools or markers of protein loss from increased vascular permeability (serum albumin and stool α_1 -antitrypsin). However, the future may bring radical changes in our ability to study the gut-associated immune system. New capsules are available that trans-

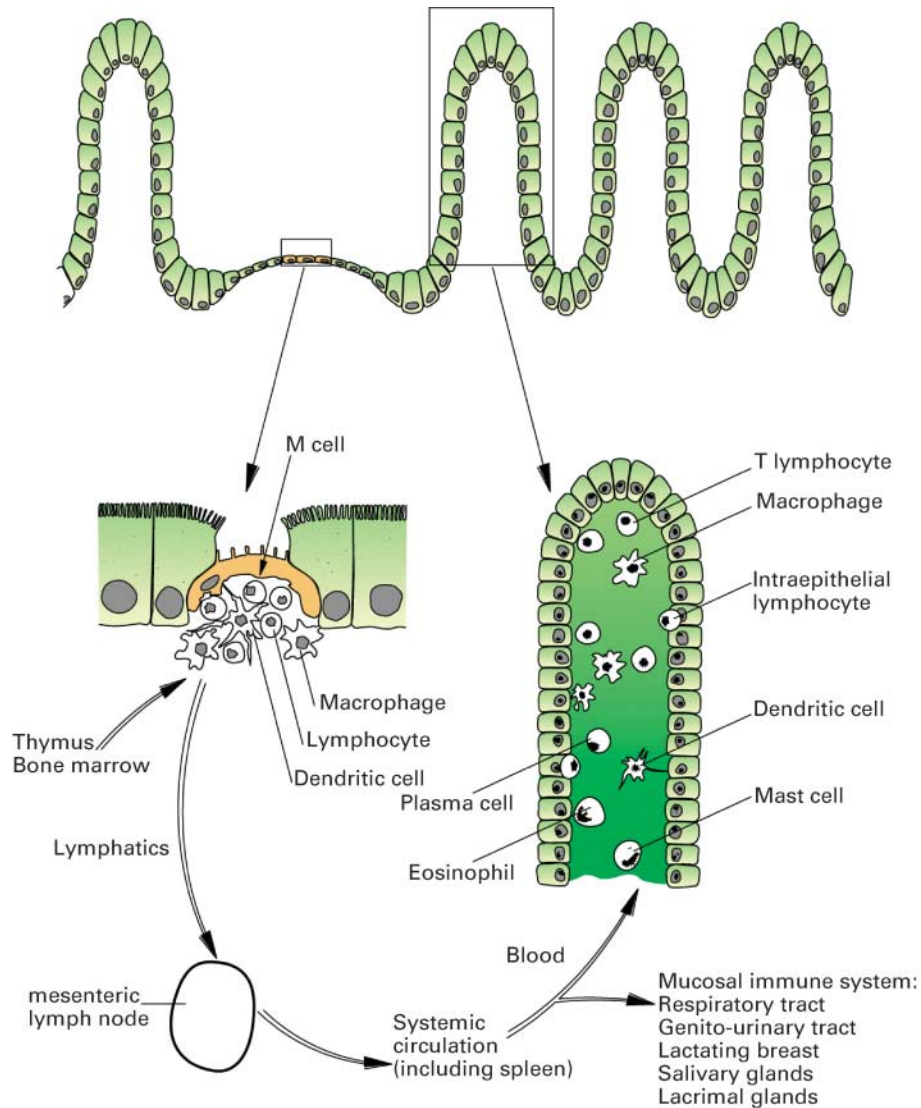


Fig. 1. Diagram of the mucosal immune system. Lymphoid cells arrive from the bone marrow (B cells) or thymus (T cells) via the systemic circulation and enter the Peyer's patches or isolated lymphoid follicles. Luminal antigen enters these organised lymphoid structures through specialised epithelium containing M cells and is presented to lymphocytes by a variety of antigen-presenting cells. These activated lymphocytes leave the follicle via the mesenteric lymphatics and enter the systemic circulation from where they selectively migrate back to the gastrointestinal tract and to other mucosal effector sites. In the intestine, the two major populations consist of lamina propria lymphocytes and intraepithelial lymphocytes. Lymphocytes in both of these compartments interact with a wide variety of other cell types. Although the small bowel is illustrated here, similar gut-associated lymphoid tissue follicles, migration patterns and effector compartments apply also to the colon. (From Dean & Elson, 1997.)

mit images of the intestine as they pass down the lumen. Such capsules are currently the subject of technological development. Once these can sample tissue, the way will be open to monitor intestinal immune system in a detailed fashion with techniques that cause a minimum of discomfort.

Questionnaires

Self-reporting of symptoms of URTI using questionnaires has been used in a number of studies designed to evaluate the effects of chronic psychological stress, exercise or nutritional supplement intervention on infection incidence (e.g. Smith *et al.* 1989; Nieman *et al.* 1990; Chandra, 1992; Meydani *et al.* 1997; Graat *et al.* 2002; Hamrick *et al.* 2002). However, this approach

leaves such studies open to the criticism that the reporting of symptoms (e.g. sore throat, runny nose, congestion, fever) is subjective and that factors other than infection (e.g. allergies, inhalation of air pollutants) could also cause some of these symptoms. Of course, infection risk not only depends on immune system status but also on the degree of exposure to pathogens and the experience of previous exposure. The average incidence of URTI in adults in developed countries is one or two episodes per individual per year (Matthews *et al.* 2002) and URTI incidence is higher in the winter months. Thus, such studies require large cohorts of subjects (e.g. fifty or more in placebo and treatment groups) who are followed over a sufficiently long time period (e.g. longer than 6 months) in order to detect potential differences in infection incidence due to a nutritional intervention.

Questionnaires may also be used to evaluate duration and severity of episodes of symptomatic illness and similar limitations apply to these. Questionnaires can also incorporate general questions such as number of days off work, and use of medications such as antibiotics. Questionnaires should also be used to record any adverse effects of a nutritional intervention. For example, in a placebo-controlled trial that investigated the effects of zinc gluconate supplements in the treatment of acute URTI, nausea and bad taste reactions were reported by 50% of the subjects taking Zn (Smith *et al.* 1989).

In studies using postal questionnaires there may be a response bias. For example, in the study by Nieman *et al.* (1990), on infection incidence following a marathon race only 47% of questionnaires were returned, and the respondents may have been mainly those who developed symptoms. Thus, it is preferable that infections are clinically confirmed rather than self-reported. The presence of an infection verified by the isolation of a virus or bacterium from body fluid samples or an increase in the pathogen-specific antibody titre would be the gold standard in this regard. The combined use of good questionnaires and immunological markers in an intervention study can help to better understand the clinical significance of changes in the measured immunological markers. In several studies (e.g. Chandra, 1992; Meydani *et al.* 1997), the results from questionnaires supported the immunological data. Questionnaires are particularly desirable if *in vivo* assays of immune function cannot be included in a study.

Emerging techniques and markers

Our present knowledge of markers of the immune system is inevitably based on our current understanding of immunology and on the techniques available. It is likely that future work will identify new immune markers that are better related to subtle changes in resistance to infections.

Innate immunity

Recent years have seen a resurgence of interest in the innate immune system (Beutler, 2004). Current knowledge is limited to the identification of components of the immune system that are involved and of their basic interaction with pathogens. Little is known about how alterations in the components of the innate immune system relate to the ability to fight infections.

New information about the recognition of bacterial pathogens by the innate immune system (i.e. pattern recognition receptors) has become an active field of study over the last 5 years since the discovery of TLR (see p. 454). Intracellular molecules (Nod proteins) are also able to recognise other parts of the bacterial cell wall once bacteria have invaded (Girardin & Philpott, 2004). At present, we have no information as to how the degree of variation in the expression of these receptors may affect resistance to infection. If there is a functional effect of variation, then nutritional modulation of such variation would be important.

Defensins are a series of molecules that can lyse bacteria and other cellular pathogens (Ouellette & Bevins, 2000). They are produced by a number of different cells including neutrophils, paneth cells and epithelial cells. A series of families of defensins, based on their amino acid structure, exist. Again, future work is needed to establish how expression of the defensins is related to

resistance to infection and the possibility that nutrients may alter defensin regulation is an exciting possibility.

Adaptive immunity

Markers that orchestrate immune function are obvious candidates for studies on resistance to infection. Two sets of T cells are currently the topic of intensive study and may well lead to useful markers in the future (for reviews see Curotto de Lafaille & Lafaille, 2002; Walker, 2004). Recent interest has also centred on the question of finding a marker to identify recent thymic emigrants (Ye & Kirschner, 2002). A variation in the production of T cells from the thymus might be a useful mechanism of marking immune function, if this were proven to be important in the resistance to infection. Lack of a marker to identify recent thymic emigrants is a hurdle to this, but an assay measuring T cell receptor excision circles has been used as a marker. There is evidence that their concentrations may change during normal ageing and during HIV-1 infection (Touloumi *et al.* 2004). However, this is still a very new field.

Gut immunity

Wireless capsule endoscopy. An obvious problem for the assessment of the mucosal immune system is the difficulty in accessing material from the intestine. Endoscopy is a well-validated technique for diagnosing disease and monitoring treatment, but the ethics of performing endoscopy on healthy volunteers is controversial. The use of capsules that pass the gastrointestinal tract (Fritscher-Ravens & Swain, 2002; Mylonaki *et al.* 2003; Swain, 2003) may result in new techniques that resolve this problem. Currently, the wireless capsule is a visual relay device that transmits images of the gastrointestinal tract from inside the intestine to a receiver in the subject's jacket. This in turn is used to download a real-time video picture of the surface of the gastrointestinal tract. While such techniques are useful for identifying morphological variations, they are currently limited in their ability to examine the mucosal immune system. Further developments for this will be needed. The first is the ability to control the capsule by the investigator rather than being dependent for its movement on peristalsis. The use of a motor that controls an external caterpillar track around the capsule is one way forward. More problematic is the ability of the capsule to sample material. There are two unresolved areas: the sampling device and the internal storage. In the traditional Crosby capsule used for single duodenal biopsy, these problems were resolved by the rotation of an internal knife that was released by vacuum. It is possible that such adaptations can be utilised in the free-flowing capsule but considerable technical problems remain.

Criteria for the evaluation of immune markers

Clinical symptoms of infection (e.g. presence, duration and severity of fever or diarrhoea, use of antibiotics) are sometimes used in human trials as an indirect marker of immunocompetence, but such studies typically require a long study period and a large number of subjects. In many situations this is not compatible with the possible size or length of nutrient intervention trials and *ex vivo/in vitro* immune markers are used instead to assess the effects of interventions. However, these markers have to be

chosen carefully for the findings to be relevant and of use. A set of criteria has been established here that allows the different markers described above to be evaluated (Table 5). These criteria are 'biological relevance', 'biological sensitivity' and 'feasibility'.

Biological relevance

Ideally, immune markers should correlate with relevant clinical endpoints and predict resistance to infection and other illnesses associated with dysregulation in immune function. The association between changes in any given immune marker and predisposition to, or presence of, a disease should preferably be known. Thus, markers that are differentially expressed in normal and high-risk or diseased individuals are especially meaningful. Nevertheless, the relationships between *ex vivo/in vitro* responses and *in vivo* realities are often quite difficult to establish. In order to understand the biological consequences of changes in immune markers measured *ex vivo/in vitro* and the relationship of such changes to health, long-term observational studies have to be initiated.

An immune marker could be involved in more than one aspect of the immune response to infection. Experimental, perhaps mechanistic, data that support the biological function of a marker are important to confirm that this marker is specifically involved in the biological process being studied. When a single immune marker is not specific to a particular component of the immune response, then it is essential to use a battery of markers.

Biological sensitivity

For any immune marker there will be some degree of variation both within and between subjects (Cummings *et al.* 2004). There are many subject-specific and technical determinants of variability in immune markers and these should be controlled as far as possible (see p. 479). For instance, immune markers are affected by differences or changes in physiological state (e.g. sex, gender, age, menstrual cycle, physical exercise, nervous stress, fed *v.* fasted, alcohol intake, smoking habits) or they may exhibit changes during the day (Liebmann *et al.* 1998; Haus & Smolensky, 1999) or with season (Nelson & Demas, 1996; Mann *et al.* 2000; Myrianthefs *et al.* 2003; Nelson, 2004). If these factors are not properly controlled it may be difficult to interpret changes in immune markers following a dietary intervention. In the absence of suitable controls, nutrient-induced changes may not be sufficiently large to allow their identification against the background of the physiological or biological variation.

The sources of variability have to be taken into account when determining the exclusion and inclusion criteria that are used to identify which individuals can participate in an intervention study. The variation between subjects allows the statistical distribution to be determined and will influence decisions about the number of subjects needed in order for the study to be sufficiently powered to identify a statistically significant effect of an intervention. If there are considerable variations in an immune marker, study designs should include measures that are performed both before and during the intervention period within the same subjects to control for this variability. Where possible, placebo-controlled, randomised cross-over designs are preferable, so long as there are no carry-over effects between intervention periods.

Feasibility

Immune markers should be determined by validated assays that are specific, sensitive, reproducible and robust. Measurement of many of the immune markers described earlier requires immediate processing of blood, which might involve purification and subsequent counting and culture of cells under sterile conditions. Whilst most laboratories will have access to the necessary equipment items (laminar flow cabinet, centrifuges, cell counter, CO₂ incubator), measurement of other markers requires the availability of flow cytometry facilities, which may be limited in some settings. Some immune markers (e.g. cytokine production by cultured mononuclear cells) require immediate preparation and culture of cells under standardised conditions, followed by measurement of the marker in the culture supernatant. In such conditions, supernatants are typically stored frozen (preferably at -80°C) for later batch analysis. The real-time processing of large numbers of blood or cell samples can create logistical difficulties and these need to be considered when planning an intervention study. Thus, for a clinical trial with a large number of enrolled subjects it may be advantageous to exclude markers that are technically or logistically difficult to measure. Cryopreservation of cells for later assessment of function is a possibility for some markers.

Recommendations for the use of immune markers

The strengths and weaknesses of the different immune markers that are currently available have been evaluated based upon the criteria described previous. Three categories of suitability have been established: high, medium or low (Table 5).

Four highly suitable immune markers have been identified. These are vaccine-specific serum antibody production (see p. 460), the DTH response (see p. 467), vaccine-specific or total secretory IgA in saliva (or other relevant fluids; see p. 471) and the response to attenuated pathogens (see p. 471). These are considered highly suitable because they measure an integrated *in vivo* response to an immune challenge of some sort. As such, they are each biologically relevant, with an identified association to the clinical endpoint (i.e. a robust host defence against pathogens) and with lower expression in individuals who are more susceptible to infections. The unavailability of the commercial kit for the DTH test will limit the widespread use of this immune marker in the future. However, new approaches to the DTH test are emerging (see earlier) that will most likely allow for the continued measurement of this marker, although this may be confined to fewer laboratories. The response to attenuated pathogens is also an emerging immune marker, but this marker may require special ethical considerations (see p. 459).

A number of *ex vivo* immune measurements are available, these giving information about the functions of circulating phagocytes, NK cells, APC and lymphocytes (see p. 467). These measurements may be used to provide mechanistic understanding of the effect of an intervention on highly suitable markers. When measurement of a 'preferred' *in vivo* marker is not possible, the *ex vivo* markers may be used to assess immune functions. However, it should be noted that these markers are individually considered to be of medium or low suitability (Table 5). This assessment is based largely upon the lack of clear association between a change in an *ex vivo* immune marker and a change in

Table 5. Immune function markers scored for their usefulness

	Marker	Biological relevance*			Biological sensitivity†			Feasibility‡			Marker score\$	Remarks
		1	2	3	1	2	3	1	2	3		
		1	2	3	1	2	3	1	2	3		
Systemic immune markers <i>In vivo</i>	Response to vaccination	++	++	++	na	++	++	++	++	++	H	Medical supervision needed
	DTH	++	++	++	++	+	++	++	++	++	H	Requires <i>in vivo</i> measurement 24–48 h after application
<i>Ex vivo</i> innate	Phagocyte function	+	+	+	++	++	++	++	++	++	L	More sensitive than phagocytosis
	Killer cell activity	++	+	+	++	++	++	++	++	++	M	
	APC function	+	+	+	++	++	++	++	++	++	M	Recommended panel: IL-1β, TNF-α, IL-6, IL-12
	Lymphocyte function	+	+	+	+	+	+	+	+	+	L	Preferred to combine polyclonal stimulation with antigen-specific stimulation. Variability should be reported Recommendation: CD69 early activation marker. Preferred to thymidine
Basal markers	PGE ₂ production	-	-	-	+	-	+	+	+	+	L	
	HLA-DR expression	+	+	+	+	+	+	+	+	+	M	
	Proliferation	++	++	++	?	?	?	?	?	?	M	Recommended panel: IFN-γ, IL-2, IL-4, IL-5, IL-10, TGF-β
	Activation marker expression	?	?	++	++	+	++	++	++	++	L	Recommended panel: IFN-γ, IL-2, IL-4, IL-5, IL-10, TGF-β
Circulating factors	Cytokine production	++	++	++	++	+	++	+	++	++	M	
	Antibody production	+	+	++	+	-	+	+	++	++	M	
	Complement components	?	+	+	+	++	++	++	++	++	L	
	Ig	+	+	++	+	++	++	++	++	++	L	Not a marker of immune function but a non-specific indicator of inflammation or infection
Circulating leucocytes	CRP	+	+	-	++	++	++	++	++	++	L	
	Pro- and anti-inflammatory cytokines (and cytokine receptors)	?	-	-	?	?	?	+	+	+	L	
	Total blood count	+	+	++	++	++	++	++	++	++	L	Helps to interpret functional <i>ex vivo</i> markers
	Lymphocyte subsets	+	+	++	++	++	++	++	++	++	L	Helps to interpret functional <i>ex vivo</i> markers
Mucosal immune markers Gut associated immune function	Sugar permeability	-	-	-	+	+	+	+	+	+	L	Does not directly assess immune function. Only changes under physical stress or at the extremes of age. There does not seem to be any effect on mucosal barrier function in healthy subjects
	Bacterial permeability	-	-	-	-	-	-	+	+	+	L	
	Serum endotoxins	-	-	-	-	-	-	?	?	?	L	
	Vaccine-specific sIgA	+	+	+	+	+	+	+	+	+	H	
Salivary and stool Ig	Non-specific sIgA	+	+	+	+	+	+	+	+	+	H	
	Stool calprotectin	-	-	-	?	?	?	+	+	+	L	Disease marker

Table 5. Continued

Marker	Biological relevance*			Biological sensitivity†			Feasibility‡			Marker score §	Remarks
	1	2	3	1	2	3	1	2	3		
Faecal water cytokine concentration	-	-	-	+	?	-	+	+	-	L	Disease marker
Response to attenuated pathogens	N/A	+	+	+	+	+	+	+	+	H	Ethical committee may ask for specific information

DTH, delayed-type hypersensitivity; APC, antigen-presenting cell; Ag, Antigen; NK, natural killer; PGE, prostaglandin E; HLA, human leucocyte antigen; CRP, C-reactive protein; sIgA, secretory IgA; IFN, interferon; TGF, transforming growth factor.

+, +, Criterion met with confidence; +, criterion met; -, criterion not met; ?, insufficient data to evaluate criterion; N/A, not applicable.

* Criteria: 1, differentially expressed in normal and high-risk/diseased individuals; 2, correlates with relevant clinical endpoint; 3, known mechanistic link.

† Criteria: 1, low within-subject variability; 2, low between-subject variability.

‡ Criteria: 1, validated assay available; 2, robustness of assay; 3, technical feasibility.

§ Overall usefulness of marker: H, high; M, medium; L, low.

susceptibility to infection. Nevertheless, some of the *ex vivo* immune markers are sufficiently reliable, sensitive and feasible to support their measurement in human intervention studies. In particular, this applies to measurement of NK cell activity and to phagocyte oxidative burst. Furthermore, a combination of tests representing the function of one type of immune cell or one component of the immune response would be a significant advantage over single marker measurements and is recommended. Indeed, it may be preferable to use several combinations of *ex vivo* markers. Such combinations might include T cell proliferation, expression of early and late activation markers on the surface of T cells and production of key TH1-, TH2- and regulatory type T cytokines (e.g. IFN- γ , IL-2, IL-4, IL-5, IL-10 and TGF- β) as an overall assessment of T lymphocyte function, or surface expression of HLA and production of key immunostimulatory cytokines (e.g. IL-1 β , IL-12) as an overall assessment of APC function. The production of antigen-specific antibodies by cultured lymphocytes taken from individuals sensitised to the antigen (e.g. via vaccination) was considered to be a good measure of B cell function. The use of combinations of immune markers each individually considered to be of medium suitability would greatly increase the confidence in any findings from an intervention study, particularly if consistent effects were seen across the range of related markers. In addition, the combination of markers can provide mechanistic information that might be missed if only single *ex vivo* markers were measured.

A few of the *ex vivo* markers are considered to be of low suitability, largely because of the lack of clear association between a change in the marker and a change in susceptibility to infection.

Most of the *ex vivo* markers referred to earlier and in Table 5 may be evaluated using either whole blood or isolated PBMC, the latter being a mix of lymphocytes and monocytes (approximately 85:15). However, it must be borne in mind that the majority of immune cells are not in the blood circulation. For example, only 2% of total lymphocytes are circulating at any given time (Westermann & Pabst, 1990). Furthermore, the activity of blood monocytes might not provide a good indicator of that of tissue macrophages (Ceddia & Woods, 1999). Isolation of cells from the bloodstream allows for precise control over the number and types of cells being studied. However, isolation removes the cells from the other cell types and blood constituents that they would normally be in contact with and this may alter *ex vivo* responses compared with those that the cells might undergo *in vivo*. This places a limit on extrapolation of findings in cell culture to the whole body situation.

All basal immune markers were considered to be of low suitability (Table 5). This is because these markers, which are all measured in the bloodstream, do not typically represent an immune response and so they are not especially informative about how well the immune system will function. For example, in contrast to vaccine-specific antibody responses to vaccination, circulating Ig concentrations are not informative about how the immune system would respond to a challenge. Furthermore, concentrations in the blood compartment may not be representative of concentrations elsewhere. For example, the concentration of IgA in the blood poorly represents the secretion of secretory IgA in the gut (Delacroix *et al.* 1982). Thus, the measurement of complement components, Ig, cytokines and cytokine receptors in the bloodstream of healthy individuals is not recommended as useful or informative. Exceptions to this might be where specific stresses such as exercise (see p. 457) or eating fat-rich

meals (Burdge & Calder, 2005), which are known to elicit responses characterised by increased concentrations of inflammatory cytokines in the bloodstream, are being used.

Measurement of circulating leucocyte subsets was considered to be of low suitability with regard to its relevance as an immune marker that might be sensitive to dietary intervention. Despite this, it is strongly recommended that differential leucocyte counts and identification of mononuclear cell subsets (T, B, CD4⁺, CD8⁺, NK cells and monocytes) be routinely performed in any intervention study in order to obtain valuable information about the circulating immune cell status of the subjects being studied.

Circulating CRP concentration was also considered to be of low suitability as a marker of immune function. In healthy uninfected individuals CRP concentrations are low but these will increase greatly upon infection. Thus, CRP is not a measure of immune function but of the response of the host to infection. It may be that in some settings CRP concentrations could be used as an outcome indicating the presence of infection. It should also be noted that concentrations below the previously recognised lower threshold of clinical significance (10 mg/l) are now known to vary among individuals and these low concentrations are positively associated with cardiovascular risk (Albert & Ridker, 1999).

A number of gut-associated markers were also considered of low suitability. Some of these (sugar and bacterial permeability; serum endotoxin) are not immune markers *per se* but rather markers of intestinal integrity. Likewise, the concentrations of cytokines and other proteins in faeces or faecal water are indicators of inflammatory activity rather than of immune competence, although these may be targets for anti-inflammatory strategies.

In summary, there is no single immune marker that accurately reflects an individual's resistance to infection and the best measure still is the clinical outcome after infections (incidence, severity and duration of the disease). However, there is a range of *in vivo*, *ex vivo* and blood measurements that can be made that reflect, to differing extents, an individual's immune competence. These markers have been evaluated according to several criteria and four *in vivo* markers were identified as highly suitable for use in human intervention studies. In addition, combinations of several *ex vivo* immune markers representing the activities of key cells of the immune system were identified as being of medium suitability. Measurements of circulating proteins and of gut integrity were identified as being of low suitability as immune markers. It is recommended that the *in vivo* markers of systemic or gut-associated immunity be used where possible. These measurements can be supported by the selected, relevant *ex vivo* markers (NK cell activity, phagocyte oxidative burst, T cell function, B cell function, APC function). This will be of mechanistic value since it will provide an understanding of how an effect on the *in vivo* marker occurred (or not). In the absence of the *in vivo* markers, the full combination of *ex vivo* markers should be used. These recommendations are based upon the current knowledge of the immune system and upon the technologies currently available. It is possible that new immune markers or technologies will become available in the future and these might result in modifications of these recommendations. In order to improve our knowledge in that field, it would be valuable to include a panel of immune markers in the coming clinical trials, as

there is a need for data correlating changes in markers with changes in clinical endpoints.

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