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Article

Accepted Version

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Keywords: Ageing, fatty acid, immunity, nutrition, probiotic

Abbreviations used: AMPK, AMP-activated protein kinase; AA, arachidonic acid; CMV, cytomegalovirus; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; mTOR, mechanistic target of rapamycin; PBMC, peripheral blood mononuclear cells; PUFA, polyunsaturated fatty acid; TCR, T-cell receptor.

Abstract

Immunosenescence during ageing is a major challenge, weakening the ability of older individuals to respond to infection or vaccination. There has been much interest in dietary strategies to improve immunity in older people, but there is an assumption that modulation of the immune response in older people will be based on the same principles as for younger adults. Recent evidence suggests that ageing fundamentally alters the impact of nutrition on immune function. As a result, interpretation of data from studies investigating the impact of diet on immune function is highly dependent on subject age. Study design is critically important when investigating the efficacy of dietary components, and most studies involving older people include rigorous inclusion/exclusion criteria based on medical history, laboratory tests, general health status, and often nutritional status. However, immunological status is rarely accounted for, but can vary significantly, even amongst healthy older people. There are several clear examples of age-related changes in immune cell composition, phenotype and/or function, which can directly alter the outcome of an intervention. This review uses two case studies to illustrate how the effects of n-3 polyunsaturated fatty acids and probiotics differ markedly in young vs older subjects. Evidence from both suggests that baseline differences in immunosenescence influence the outcome of an intervention, highlighting the need for detailed immunological characterization of subjects prior to interventions. Finally, future work elucidating alterations in metabolic regulation within cells of the immune system as a result of ageing may be important in understanding the impact of diet on immune function in older people.

Introduction

Nutritional status has a profound influence on resistance to infection, which is exemplified by the vicious cycle between undernutrition and infection in developing countries [1]. However, vulnerable groups in developed countries are also at risk of age- or disease-related malnutrition, which can impact on the immune response to infection and to vaccination. Thus, while decreased immune function due to malnutrition primarily affects children in developing countries, in the developed world, it is mainly a problem for older people [2]. By 2050, approximately 25% of the population will be older than 65 years [3] and the impact of this on public health is a major global challenge. However, decreased immune function as a result of malnutrition should not be confused with immunosenescence; an obvious difference is that malnutrition and, to some extent its consequences, are treatable. Immunosenescence is irreversible and describes the biological ageing of the immune system, which is associated with a progressive decline in both innate and adaptive immunity, poor response to vaccination and increased prevalence of cancer, infections and autoimmune and chronic diseases. While nutritional interventions may delay this process, the evidence for this remains controversial, particularly in terms of the nature and potency of immunomodulatory activity and of translation into a corresponding change in clinical outcome [4; 5]. Furthermore, there is a fundamental lack of understanding as to how immunosenescence alters the response of cells of the immune system to dietary components. Most studies examining the effects of diet on immune function fail to adequately characterize target populations in terms of nutritional status, health status, genetic background and few, if any, characterize them in terms of immunological status. This review focuses on two case studies, which demonstrate that failure to account for immunosenescence can significantly influence the outcome of a nutritional intervention. It also explores proposed mechanisms by which ageing alters

metabolic regulation of immune cells and whether metabolic pathways could be targeted for immunoregulation.

Case study: ageing alters the immune response to n-3 polyunsaturated fatty acids

Fatty acids play diverse roles in all cells, serving as an important source of energy, as structural components of cell membranes, signaling molecules, bioactive mediators and regulators of gene expression. Human immune cell phospholipids contain about 1% eicosapentaenoic acid (EPA) and 2.5% docosahexaenoic acid (DHA) in addition to 20% arachidonic acid (AA) [6; 7]. As the long chain n-3 polyunsaturated fatty acid (PUFA) content of the diet increases, lymphocyte AA decreases in a curvilinear fashion. In human studies, dietary n-3 PUFA never exceeds 3 % of total energy, whereas in animal studies, intake is often considerably higher, and this is thought to explain the discrepancies that exist between animal and human studies investigating the immunomodulatory effects of n-3 PUFA [7]. As a result, it remains unclear to what extent and at what dose n-3 PUFA have immunomodulatory effects in humans. Nevertheless, the literature suggests that fish oil has a greater impact on immune function in elderly compared with young subjects [8; 9; 10] and that this may be related to the fact that older subjects appear to incorporate EPA into plasma and PBMC more readily than younger subjects [11] (**Figure 1**). EPA resulted in a dose-dependent decrease in neutrophil respiratory burst in older, but not younger subjects [11]. However, PGE_2 production by PBMC was decreased in both groups and phagocytosis and cytokine production were not affected in either group [11]. This highlights the fact that age is likely to be an important factor when considering the impact of n-3 PUFA on immunity, not only because of the influence of immunosenescence, but also because immune cells from older subjects appear to be more responsive to the availability of n-3 PUFA. Recent work suggests that the cholesterol content of T lymphocytes from healthy elderly subjects is higher than that of young subjects, and that

membrane fluidity is subsequently decreased [12]. Furthermore, the coalescence of lipid rafts at the site of T cell receptor engagement is impaired in elderly subjects [12; 13]. The impact of ageing on lipid raft composition and function appears to be most evident in the $CD4^+$ T cell population and affects cytokine signaling [13; 14]. Thus, the greater responsiveness of T cell membranes to n-3 PUFA in older subjects could result in alteration of lipid raft structure, and subsequently of cell function, effects which are absent in younger subjects.

Case study: ageing alters the immune response to probiotics

Influenza is a major cause of death in older people and while vaccination offers a prophylactic solution for preventing infection and associated complications, immunosenescence significantly impairs vaccine efficacy [15]. Potential adjuvants and dietary strategies to improve the immune response to influenza vaccines are therefore of interest, particularly in older people. Emerging evidence suggests that the resident gut microbiota plays an influential role in shaping antiviral defenses and modulating the outcome of viral infections through inflammasome-mediated cytokine release [16], Antibiotic-treated mice have reduced levels of interleukin-1ß (IL-1β) secretion in the lung during influenza infection, supporting the suggestion that gut-resident bacteria support cytokine production [16]. It has been speculated that gut microbes release low levels of pattern recognition receptor ligands, which provide signals for inflammasome-mediated cytokine release (for example, in the lung during influenza infection). These in turn regulate the activity of respiratory dendritic cells during activation of adaptive immunity against the virus [16], and together, this forms the basis for the hypothesis that pre- and probiotics may modulate responses to infection or vaccination.

Trials investigating the use of probiotics in prevention of common respiratory illnesses have produced mixed results [17], although a recent systematic review concluded that they

significantly reduce episodes of acute upper respiratory tract infection and antibiotic usage in infants and young to middle-aged adults [18]. Response to vaccination is increasingly being used as a surrogate for the response to infection [19]. The majority of studies investigating the impact of probiotics on responses to vaccination have been conducted in healthy adults, and some show borderline effects of probiotics on serum or salivary IgA titres, although the clinical relevance is not clear [20]. Studies in infants and in elderly subjects, particularly those examining the response to influenza vaccination, are very limited, as are studies on the effects of prebiotics on immune function [21] and vaccination [20]. Since ageing is associated with reduced biodiversity and compromised stability of the gut microbiota [22], as well as immunosenescence, older individuals may derive particular benefit from intervention with pre- and/or probiotics.

Previous studies investigating the effects of probiotics on the response to vaccination have mainly focused on antibody production. While some studies have reported a modest effect of probiotics on the antibody response to vaccination in adults, trials in older subjects are largely inconsistent and data are limited [20]. In a recent study (the PRobiotics, IMmunity and AGEing; PRIMAGE trial), we demonstrated that while there was marked impairment of the antibody response to influenza vaccination in older subjects, intervention with a novel synbiotic, *Bifidobacterium longum bv. infantis* CCUG 52486 combined with gluco-oligosaccharide (*B. longum* + Gl-OS) failed to reverse this impairment [23]. Although there is general consensus that ageing impairs the response to influenza vaccination [24], there are very few robust studies specifically comparing responses of young and older subjects, and there are no other studies directly comparing the efficacy of pre- and probiotics on the immune response of young and older subjects to vaccination. In the PRIMAGE trial, the response of the young and older subjects to the intervention differed to some degree. In older subjects consuming the synbiotic, there was a trend for reduced seroconversion to the Brisbane

subunit of the vaccine, whereas in the young subjects, there were trends for enhanced production of vaccine-specific IgM and, to some extent, IgG [23]. Increased production of vaccine-specific IgM and IgG following intervention with probiotics has been reported in several other studies [25; 26; 27; 28; 29]. The possibility that there is a differential immune response to probiotics in young vs older subjects has also been demonstrated in *in vitro* studies. You *et al.* [30] demonstrated that peripheral blood mononuclear cells (PBMC) from older subjects (60-85y) were more responsive to the immunoregulatory effects (IL-10 induction) of two strains of bifidobacteria than young subjects (18-30y), whereas PBMC from young subjects were more responsive to the immunostimulatory effects (IL-12 induction) of two strains of lactobacilli. Further studies demonstrated that probiotics increased the responsiveness of DCs in older subjects to a greater degree than young subjects, but this was not sufficient to overcome the impact of immunosenescence in a mixed leukocyte reaction [31]. The choice of probiotic, particularly for older individuals, is a matter of debate and it has been suggested that 'successfully aged' donors of probiotic strains might survive better in an older host and achieve a more suitable equilibrium with the resident microbiota [32]. *Bifidobacterium longum bv. infantis* CCUG 52486 is an example of a strain present in particularly healthy subjects aged >90y [33]. It has subsequently been demonstrated to have particular ecological fitness and anti-pathogenic effects *in vitro* [34] and, as described above, immunomodulatory effects which are strongly influenced by the age of the host [30; 31]. Further immunological characterization in the PRIMAGE trial revealed that B and T cell profiles differed markedly between young and older subjects, and that vaccination increased numbers of specific memory subsets in young subjects, but failed to do so in older subjects (Enani et al., unpublished data). A key finding was the observation that there was a greater degree of immunosenescence at baseline in older subjects randomized to the synbiotic, which occurred entirely by chance, but could explain the particularly poor response of these subjects

to the vaccination [23]. T cells are particularly susceptible to senescence, resulting in loss of CD28; repeated antigenic exposure, for example to cytomegalovirus (CMV), is suggested to play a major role in this [35; 36]. Latent infection with CMV has been demonstrated to result in a poor response to infection and vaccination [36]. In the PRIMAGE trial, not only did older subjects randomized to the synbiotic have a significantly higher number of senescent ($CD28^-$ $CD57^+$) helper T cells at baseline compared with those randomized to the placebo, they also had significantly higher plasma levels of anti-CMV IgG and a greater tendency for CMV seropositivity. Moreover, higher numbers of $CD28^-CD57^+$ helper T cells were associated with failure to seroconvert to the Brisbane subunit of the vaccine, strongly suggesting that the subjects randomized to the synbiotic were already at a significant disadvantage in terms of likely ability to respond to the vaccine compared with those randomized to the placebo and that differences in immunosenescence between the randomized groups at baseline may have influenced the outcome of the intervention (**Figure 2**). Future work therefore needs to consider prospective randomization of subjects based on robust immunological markers; this is challenging given the wide range of potential markers and uncertainty regarding their predictive value.

Ageing alters metabolic regulation of T cells

Over the past decade, our understanding of T cell activation has extended to exploration of integration between canonical T cell signalling pathways and metabolic signalling programmes [37], and it has been proposed that immunosenescence is linked to alterations or defects in that integration [38]. Although several transcription factors and serine/threonine kinases are central to the integration of immunological and metabolic pathways [37], the energy sensor, AMPK, is of particular interest in the context of ageing. AMPK is a central regulator of metabolic stress and is activated by an increase in the AMP/ATP ratio, as well as

by T cell receptor (TCR) engagement. In fact, it has been suggested that AMPK activation in response to antigen anticipates ATP depletion even in the presence of adequate nutrients [23]. In AMPK deficient T cells, metabolic stress due to glucose deprivation induces enhanced cell death. Senescent T cells demonstrate spontaneous phosphorylation- and therefore activation- of AMP [38]. However, contrary to expectation, senescent cells did not contains low levels of ATP [8; 38]. Instead, it is suggested that AMPK activation triggered by glucose deprivation results in activation of the p38 pathway, which leads to DNA damage and immunosenescence [38]. Conversely, AMPK silencing restores proliferation [37]. This is a previously unrecognized mode of activation for p38 in T cells and the first demonstration of a pathway which integrates low nutrient sensing with DNA damage and senescence. The observation that nutrient deprivation triggers pathways linked with immunosenescence seems to contradict the widely-held belief that caloric restriction enhances life span, but data on caloric restriction and infections is not clear cut and this remains an important area for future work.

Transcription factors and signalling proteins involved in regulatory and metabolic pathways represent novel targets for immune modulation. Indeed, it has been suggested that targeting AMPK and mTOR may be a strategy for suppressing immune responses and treating inflammatory diseases [37]. However, the suggestion that this may allow more selective regulation of immune responses than ubiquitous signalling pathways should be interpreted with caution as there is no clear reason to believe that this is the case.

Concluding remarks

Ageing alters the immune response to dietary interventions; specific examples described in this review demonstrate that young and older subjects respond differently to interventions involving dietary fatty acids and probiotics. It is critical that baseline differences in immunosenescence in dietary studies involving older subjects are accounted for as they can

directly influence the outcome of the intervention. Ageing also alters metabolic regulation of T cells; elucidation of alterations in metabolic regulation in ageing T cells may prove to be important in understanding the impact of diet on immune function in older people.

Acknowledgements

The author declares no conflict of interest. Some of the work described in this review was supported by a grant (BB/H00470X/1) from the Biotechnology and Biological Sciences Research Council Diet and Health Research Industry Club (BBSRC-DRINC).

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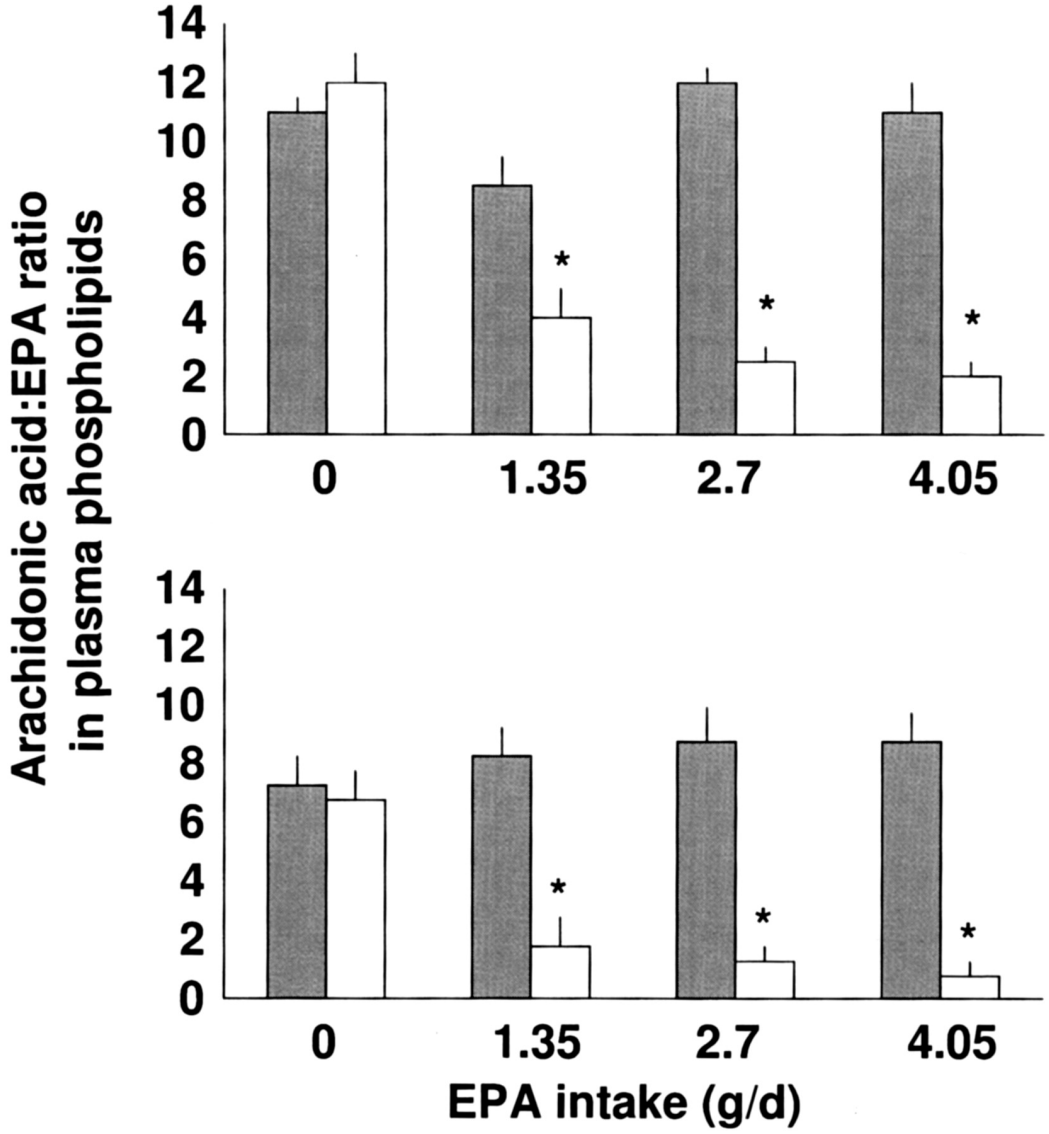

Figure 1. Arachidonic acid to eicosapentaenoic acid ratio in plasma phospholipids from young and older subjects. Mean (±SEM) ratios of arachidonic acid to eicosapentaenoic acid (EPA) in plasma phospholipids before (gray bars) and after (white bars) supplementation with placebo (0 g EPA) or low (1.35 g/d), moderate (2.7 g/d), or high (4.05 g/d) doses of an EPA-rich oil for 12 wk in the young (upper panel) and older (lower panel) subjects. n = 24, 23, 23, and 23 for the young subjects in the placebo, low-EPA, moderate-EPA, and high-EPA groups, respectively. n = 16, 16, 15, and 15 for the older subjects in the placebo, low-EPA, moderate-EPA, and high-EPA groups, respectively. At baseline there was a significant effect of age ($P < 0.001$) but not of treatment group (ie, EPA dose) and no age × treatment group interaction. At baseline the ratio was significantly higher in the young than in the older subjects ($P < 0.05$). Two-factor ANOVA showed a significant effect of treatment group ($P < 0.001$) but not of age and no age × treatment group interaction for the change in the ratio of arachidonic acid to EPA. *Significantly different from baseline, $P < 0.001$ (paired Student's t test). Figure taken from [11], with permission.

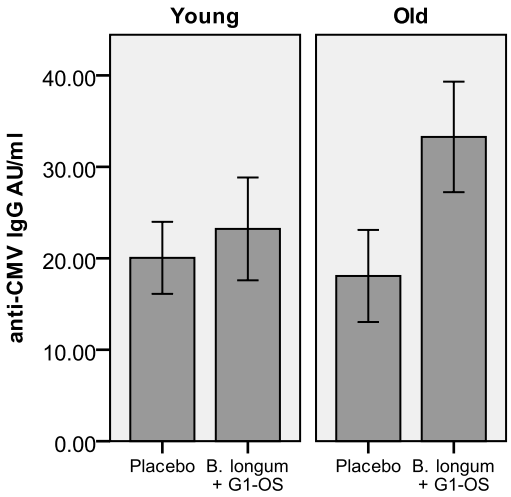

Figure 2. Baseline levels of anti-CMV IgG differ in older subjects randomized to *B. longum* + Gl-OS and placebo. Data are anti-CMV IgG (AU/ml) ± 2SEM for n=45 young and n=45 older subjects randomized to *B. longum* + Gl-OS or placebo. Data were analysed using Student's independent t-tests for differences between young and older subjects. * Denotes significant difference between treatment groups within age cohort ($p < 0.05$). The difference in CMV status between the cohorts may have influenced the outcome of the subsequent intervention. Figure taken from [23], published by Springer.