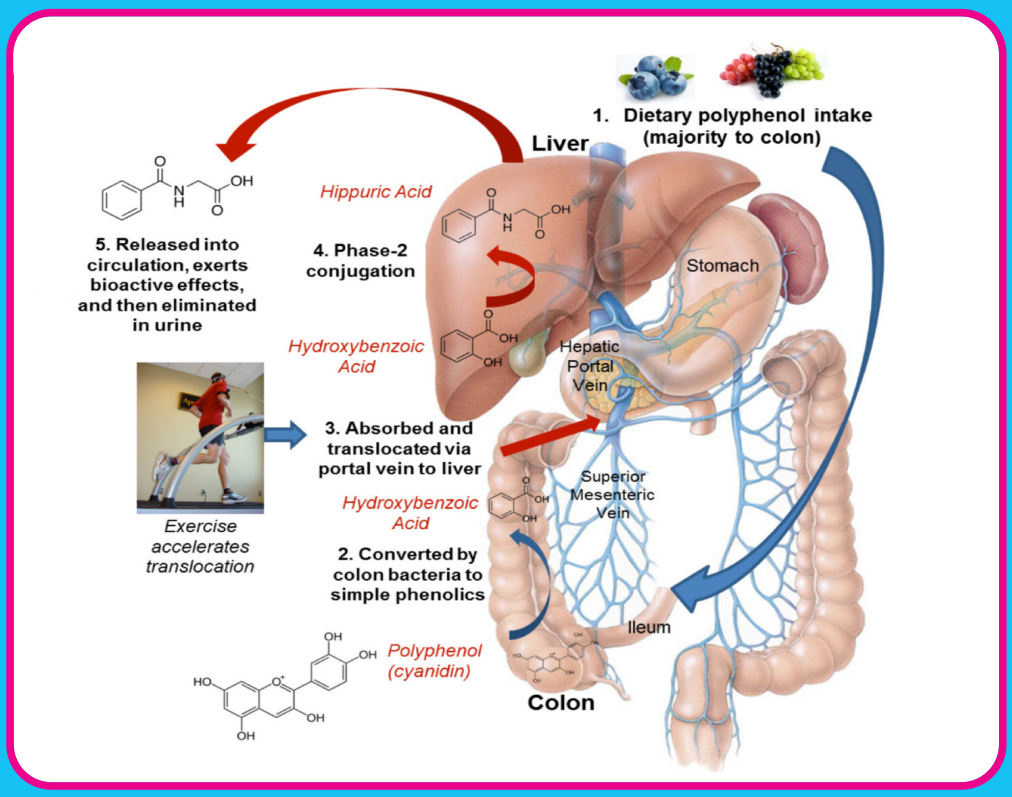
EXERCISE IMMUNOLOGY REVIEW



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EXERCISE IMMUNOLOGY REVIEW

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Contents

Editorial Statement

Exercise Immunology Review, an official publication of the International Society of Exercise Immunology and of the German Society of Sports Medicine and Prevention, is committed to developing and. enriching knowledge in all aspects of immunology that relate to sport, exercise, and regular physical ativity. In recognition of the broad range of disciplines that contribute to the understanding of immune function, the journal has adopted an interdisciplinary focus. This allows dissemination of research findings from such disciplines as exercise science, medicine, immunology, physiology, behavioral science, endocrinology, pharmacology, and psychology.

Exercise Immunology Review publishes review articles that explore: (a) fundamental aspects of immune function and regulation during exercise; (b) interactions of exercise and immunology in the optimization of health and protection against acute infections: (c) deterioration of immune function resulting from competitive stress and overtraining; (d) prevention or modulation of the effects of aging or disease (including HIV infection; cancer; autoimmune, metabolic or transplantation associated disorders) through exercise. (e) instrumental use of exercise or related stress models for basic or applied research in any field of physiology, pathophysiology or medicine with relations to immune function.

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From the Editors

This year's issue of EIR contains only four articles, which is a smaller number than usual, but one of them is a very special article: we present a consensus statement about exercise and immunonutrition, including the background, controversies and future directions in this field. We thank Stephane Bermon and Lindy Castell for initiating and coordinating this immense task, and we thank all contributing authors from across the globe, each of them as an expert in a special field, for joining in this common endeavour.

In addition to the position statement, EIR 23 includes three more articles. Gleeson et al. develop a multi-component immune model for evaluating the risk of respiratory illness in athletes. Zimmer et al. have collected and analysed available data about the effects of exercise on NK-cell cytotoxicity, including methodological issues and future perspectives. Lastly, in an invited review, Emmons and DiLisio focus on the effects of exercise on hematopoiesis and the bone marrow niche during obesity.

For EIR24 and the future, as always, we would like most contributions to be topical review articles. In the case of original research articles, we encourage the authors to embed their new data into review articles. Please note that the submission deadline for EIR24 is 31st July 2017. We hope you enjoy reading the new issue. Thank you, Rickie Simpson, Neil Walsh and Jonathan Peake, for the close and friendly teamwork. Thank you (all ISEI members), and all members of the Editorial Board for the confidence you have placed in us. We hope to see many of you at this year's ISEI meeting in Coimbra, Portugal (July 11-14).

Thank you all for your on going support of EIR. A special thanks to all the authors of EIR23 and the "Verein zur Förderung der Sportmedizin". On behalf of the Editors,

Karsten Krüger

Consensus Statement
Immunonutrition and Exercise

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Key words: Aging; Biomarkers; Exercise; Immune system; Inflammation; Nutrition; Obesity

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The co-editors (SB and LC) are very grateful to the authors for the time they have spent not only on providing appropriate articles for the series but also (together with the EIR Board) offering helpful critiques of the full document. The International Association of Athletics Federations (IAAF) is gratefully acknowledged for supporting the editing costs. The help of Anna Carlqvist in dealing with the references is warmly appreciated.

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CONSENSUS STATEMENT

The first indexed scientific publication about immunonutrition is almost 70 years old (103). Since 1947, more than 10,000 scientific articles have been published in that field representing a consistent body of knowledge. Within this field, exercise (acute or chronic) modalities are of recent interest, and only approximately 400 publications exist so far. One third of them has been published during the last three years showing that immunonutrition and exercise is a fast developing area of research. This can be explained on the one hand by the considerable development of the global sports nutrition market. On the other hand, it is also due to high levels of expectation from both elite athletes and those who are keen on the concept of "Exercise is Medicine". High level athletes are very frequently exposed to high intensity or exhausting training programmes, travel, sleep disturbances, psycho-social and environmental stressors. All these factors are potential immune disruptors sometimes leading to immunodepression and increased likelihood of illness.

In order to minimise these phenomena and to optimise recovery, nutritional interventions are often considered by athletes and their entourages as possible countermeasures to the training-related immunodepression. However, among the numer-

ous nutrients available, only a few of them have so far shown any positive effects in maintaining athlete immune health. Moreover, elderly and overweight/obese individuals who demonstrate increased inflammatory status and immune dysfunction are often prescribed physical training programmes as a countermeasure. In such circumstances, nutrition appears as a possible valuable additional support to these populations. As significant biotechnological progress has been achieved during the last fifteen years, it is of critical importance, when designing an experiment in the field of immunonutrition and exercise, to select adequate biomarkers which fit best to the research aim and the experimental design.

In this consensus statement on immunonutrition and exercise, a panel of knowledgeable contributors from across the globe provides a consensus of updated science, including the background, the aspects for which a consensus actually exists, the controversies and, when possible, suggested directions for future research.

This consensus statement series includes an introduction section (Stephane Bermon and Philip Calder) followed by sections on: carbohydrates (Nicolette Bishop); fatty acids (Philip Calder); branched chain amino acids (Eva Blomstrand); glutamine (Lindy Castell); polyphenols (David Nieman) and herbal supplements (David Senchina); antioxidants (Andreas Kavazis and John Quindry); minerals (Frank Mooren and Karsten Krüger); probiotics and prebiotics (Michael Gleeson and David Pyne); vitamin D (Graeme Close and Enette Larson-Meyer) and bovine colostrum (Cecilia Kitic). It also contains some specific sections on: immunonutrition in competitive athletes and military personnel (Neil Walsh), exercising obese and overweight (Ascension Marcos, and elderly (Simin Meydani and Dayong Wu) individuals; biomarkers used in immunonutrition, and exercise science (Neil Walsh, Simin Meydani, Dayong Wu, and Ryochi Nagatomi).

Carbohydrates are fuel for the immune cells. As far as immune functions are concerned, carbohydrates appear to be more effective when ingested during exercise rather than increasing their relative content in the daily diet. Carbohydrates have been shown to minimise some of the immune perturbations that are associated with strenuous or lasting physical exercise and can be considered as a partial countermeasure for exercise-induced immunodepression. However, carbohydrates have failed so far to demonstrate any reduction in the incidence of upper respiratory tract illness (URTI) after prolonged exercise.

There is evidence from *in vitro*, animal and epidemiological studies that several saturated fatty acids promote inflammatory processes through the omega-6 (n-6) polyunsaturated fatty acids (PUFA) and arachidonic acid pathway. n-6 PUFAs have also shown some immunodepressive effects. These phenomena occur whatever the origin of arachidonic acid: meat, eggs or plants. In untrained individuals, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) appear to decrease the exercise-induced inflammation and muscle soreness. However, most of the studies on fatty acids and immune functions and inflammation in the context of exercise have provided data which is difficult to interpret.

Similar to recommendations in sports nutrition, there is no scientific ground for an athlete to consume an excessive amount of proteins (more than 2 g/kg body weight per day) in order to boost his or her immune system or limit an exercise-induced inflammation. Despite a good rationale for glutamine (Gln) supplementation based on sound biochemical investigation, laboratory-based exercise studies have proved mainly negative in terms of providing any direct enhancement of immune function due to Gln feeding. More studies need to investigate the apparent link between glutamine and the decreased incidence of self-reported URTI. During prolonged exercise branched chain amino acids (BCAA; leucine, isoleucine, valine) are oxidized as substrates and their plasma concentration decreases. An increase in BCAA plasma concentration can help to prevent a decrease in the plasma Gln concentration, and might therefore have the capacity to influence the immune response indirectly. However, the evidence for such an effect is weak.

Zinc (Zn), magnesium (Mg) and iron (Fe) are important minerals for immune function. As these minerals often show reduced concentration or availability during exercise or training, it is important to check that the athlete's diet contains sufficient quantity of these elements. However, there is no evidence showing that supplementing non-deficient athletes might boost the immune system or prevent exercise-induced immunodepression. Selenium (Se) and manganese (Mn) cannot be classified as immunonutrients for exercise.

Prolonged, exhaustive exercise and immune system activation are associated with an increased production of reactive oxygen species (ROS), leading to a potential increase in oxidative stress. However, there is no data to support links between exercise-induced oxidative stress and immune dysfunction or the postulated benefits of dietary antioxidant supplementation in preventing immune dysfunction during exercise, or in reducing the risk of respiratory illness in athletes. Emerging evidence indicating that antioxidant supplementation mitigates important exercise-induced adaptations (including the immune system) contributes to the debate for and against antioxidant supplementation in athletes.

Herbal supplements are widely used by athletes either to improve their performance or to boost their immune system. However, few human *in vivo* studies focusing on specific immune parameters are available and most of the available studies use *ex vivo* or *in vitro* conditions. Results from these studies are conflicting and are often not in full agreement with the purported immunomodulatory claims from the food supplement industry. For example, as therapeutic immunomodulators for athletes, there is some evidence that echinacea may be efficacious whereas the evidence for ginseng is poor. Polyphenols, including flavonoids are mostly found in tea, coffee, fruits and wine. They exhibit strong anti-inflammatory, antioxidant, anti-pathogenic, and immuno-regulatory properties *in vitro.* Epidemiological data in general support that polyphenol-rich plant extracts and unique polyphenol-nutrient mixtures have small but significant effects in increasing antioxidant capacity, with inconsistent, short-term effects on mitigating exercise-induced oxidative stress, inflammation, and immune dysfunction. Quercetin consumed at high doses (500

to 1,000 mg/day) has been linked to reduced incidence of self-reported URTI in athletes.

Probiotics are interesting immunonutrients since they demonstrate immunomodulation properties on both local and systemic (some aspects of both innate and acquired immune responses) immunity. In non-athletic populations, a recent systematic review concluded that probiotic use resulted in a lower incidence of URTI, reduced numbers of illness days, and fewer days of absence from day care/school/work. Despite a lower number of studies, the same benefits seem to exist in athletic populations. Although a daily dose of ~10^{10} live bacteria is widely promoted, there is still some debate about the optimal duration of supplementation and the potential benefits of selecting and mixing specific bacterial strains with or without prebiotics.

Bovine colostrum exhibits antibacterial, anti-inflammatory and anti-viral properties. Several investigations have reported a reduction (not always statistically significant) in self-reported URTI incidence in athletes following a period greater than four weeks of bovine colostrum supplementation. However, the effect of bovine colostrum on illness duration is less conclusive.

A large number of different immune cells and functions are influenced by vitamin D. These effects are mainly mediated through modulation of the expression of several genes. Optimal circulating 25-hydroxy vitamin D concentration is possibly beyond 75 nmol/l as individuals with such a vitamin D concentration demonstrated a lower incidence of URTI than those with an actually recommended vitamin D concentration (of around 50 nmol/l). Optimal vitamin D concentrations for immune cells require further study before they can be recommended to athletes who would like to maintain their immune function at the highest level without compromising their health.

As long as the diet meets the energy demands and provides sufficient macro- and micro- nutrients to support the immune system, there is probably no need for consumption of "immune boosting" supplements. However, there are specific scenarios when elite athletes or military personnel might benefit from nutritional supplements to bolster immunity. More randomized controlled trials in these individuals with sufficient participant numbers and rigorous designs are required to investigate whether the nutritional practices adopted by elite athletes impair immunity and increase infection; and, whether purported "immune boosting" supplements benefit immune health without blunting the desired training adaptations.

Obesity is related to immune dysfunction and chronic low grade inflammation. There is a consistent body of biological evidence attesting to the anti-inflammatory effects of regular physical training in obese or overweight individuals. Indeed, regular physical activity decreases toll-like receptor (TLR)-4 expression and induces shift from M1-type macrophages to M-2 type macrophages; both of these phenomenon promoting anti-inflammatory patterns. The anti-inflammatory effects of regular exercise are also partly mediated through interleukin (IL)-6 production at the muscle level. IL-6 triggers an anti-inflammatory cascade via the induction of the anti-inflammatory cytokines interleukin-1 receptor antagonist (IL-1ra) and IL-10, and also inhibits tumour necrosis factor (TNF)-α and its associated insulin resistance pattern. IL-6 also promotes fat oxidation which is beneficial to obese individuals. These immunological changes associated with training have been proven to be clinically relevant in many studies including obese adults and adolescents.

A decrease in cell-mediated immune function in the elderly (immunosenescence) contributes to higher morbidity and mortality. Nevertheless, aging appears to be linked with an increased inflammatory response. Given the focus on exercise-induced immunodepression in this series and the journal, it seems likely that extreme exercise will exacerbate immune system impairment in aging. Moderate regular exercise, however, may even enhance immune function in the elderly. For example, calisthenic exercise increases the function of natural killer (NK) cells and T-cells in older women. It is not known whether the exercising elderly have specific nutritional needs, although many appear deficient in micronutrients essential for immune function. In addition, increased inflammation, oxidative stress and muscle damage suggest that exercising older adults might require nutrients with immune enhancing and/or anti-inflammatory properties. In terms of energy provision, total calorie intake should be adjusted to avoid conversion of excess caloric intake to body fat. Moreover, glucose tolerance and insulin sensitivity decrease with aging.

Currently, a single marker able to predict the effect of a dietary and/or exercise intervention on different aspects of immune function does not exist. The range of available biomarkers is quite wide from *in vitro* tests to clinical symptoms. However, each biomarker should be carefully chosen according to its intrinsic characteristics (links with causal pathway and clinical endpoint, biological sensitivity and specificity, feasibility, practicality, and cost) as well as the designed study's aim and primary outcome. Mechanistic studies or studies aiming at testing hypotheses at molecular, cellular or immune function levels should rather consider *in vitro* or *ex vivo* biomarkers. Whereas *in vivo* biomarkers or biomarkers relying on patient symptoms should be preferred in experiments describing integrated response or clinical studies.

INTRODUCTION: IMMUNONUTRITION, INFLAMMATION AND EXERCISE

Physical exercise (chronic or acute) influences the immune system and its functions. All immune components or functions, systemic, local or mucosal, innate or adaptive, cellular or cytokine-related are positively or negatively linked with exercise regimens (406). This body of knowledge represents the interdisciplinary field of Exercise Immunology.

Similarly, the diet (macro and micronutrients, as well as non-nutritive components) is known to influence the immune system and its functions. Quantitative aspects (from protein-energy malnutrition to unbalanced Western diets) as well as qualitative aspects (oligo elements, vitamins, mineral, anti-oxi-

dants, plant-derived immunomodulators, probiotics, amino acids, and fatty acids) can either stimulate or inhibit selective immune functions or inflammation. For instance, consumption of dietary fibres reduces chronic inflammation by decreasing lipid oxidation (124). Fibres also interact with the gut microbiota via short-chain fatty acids produced during colonic fermentation (251). Fibres from oats or barley smooth the rate of appearance of glucose in the blood, reducing the glycaemic index and glycaemic load, and as a consequence production of nitric oxide, superoxide and peroxynitrite which are powerful pro-oxidant and pro-inflammatory molecules (80). Whole-grain foods also exert anti-inflammatory properties, such as free radical scavenging, antioxidant enzyme activation, or modification of the redox status of tissues and cells (124). These close interactions between diet and the immune system are the genesis of the term "Immunonutrition" which represents another new interdisciplinary field of basic and applied research.

As the immune system and inflammation, one of its major effectors, are regulated by both exercise and nutrition, it is of particular interest to address how nutrients can affect immunity in an exercise perspective. However, when nutrition is concerned, it appears that the commitment and the goals to achieve are very different when comparing a sedentary overweight individual to a high-level athlete. Indeed, as inappropriate exercise regimens or training programmes may alter some immune functions and promote illnesses (306), elite athletes are always considering diet and nutrition plans as possible countermeasures to the so-called exercise-induced immunodepression. This latter term is more appropriate than the traditionally used term "immunosuppression" which means specific manipulation of the immune system, e.g. via cyclosporin.

However, among the numerous nutrients and foods promoted for their purported immuno-modulating effects, only a very limited number has proved to be effective in maintaining or restoring some immune functions or preventing illnesses. This consensus statement series addresses the issue of macronutrients, probiotics, vitamin D, antioxidants and plant-derived immunomodulators, minerals and some promising dietary compounds as immune support for exercising humans. It also deals with immunonutrition in exercising overweight or elderly individuals and explores the relevance of selected immune/inflammation markers commonly used when designing a nutrition study in exercise immunology.

When the diet is inappropriate (more likely excessive high glycaemic index foods and/or caloric intakes), a part of the innate immune system is overreacting to the excessive amount of visceral fat leading to a chronic, low grade inflammation and potential subsequent inflammation-related diseases. Here, regular exercise is considered as a potential countermeasure to the inflammatory-driven morbidities such as cardiovascular diseases, chronic obstructive pulmonary diseases, colon and breast cancers, insulin resistance, type II diabetes, and some neurodegenerative diseases (307,326).

The anti-inflammatory effects of exercise are achieved through several possible pathways (143). The reduction in visceral fat mass associated with a secondary reduced release of adipokines is one of the main mechanisms. Moreover, following each bout of exercise, the release of high amounts of cortisol and adrenaline associated with an increased production and release of IL 6 and other mediators now often referred to as "myokines" from working skeletal muscles contribute to the generation of an anti-inflammatory "milieu". IL-6 is pleiotropic: it may have different actions in different contexts, and thus may not always act in a manner that could be described as pro-inflammatory.

At the cellular level, a reduced expression of TLR on monocytes and macrophages and a subsequent inhibition of downstream pro-inflammatory cytokines production are observed. Within the adipose tissue quantitative and qualitative changes in monocytes-macrophages are noted. The number of M1-type macrophages is decreased as well as their associated pro-inflammatory cytokines (IL-6 and TNF-α), whereas M2-type macrophage numbers and their anti-inflammatory cytokines (IL-10 and adiponectin) are increased (205). Franceschi and colleagues (133) introduced the concept of 'inflammaging' as part of the spectrum of immunosenescence. Inflammaging is the chronic low-grade inflammatory state present in aging individuals and is believed to be a consequence of a remodelling of the innate and acquired immune system. It is characterized by increased systemic concentrations of pro-inflammatory cytokines such as IL-1, IL-6, and TNF-α (398) as well as increased C-reactive protein (CRP) concentration which are used as clinical markers. Inflammaging increases the risk of morbidities and age-related diseases, and is also associated with increased skeletal muscle wasting, strength loss, and functional impairments. In this particular context, both nutrition (115) and exercise (219) interventions are proposed for elderly or frail individuals as a countermeasure of the aging process and its associated inflammation-related diseases. This provides the rationale for producing the present consensus statements.

Most athletes, whether recreational or elite, and in all parts of the world, use sports foods and supplements. The popularity of certain types of dietary supplements demonstrates that athletes may often be more motivated by an interest in health benefits rather than, for example, direct ergogenic effects. In this series of consensus statements on immunonutrition, athletes are therefore strongly advised not only to seek the advice of a properly qualified nutritionist before embarking on supplementation but also to pay careful attention to the importance of the recommended daily allowance (RDA). It is a common misconception among athletes that, if x g of a product works, then taking double that amount will be even better! In fact, this approach is very likely to lead to health problems rather than to solutions. Readers can find more detailed discussion on this topic in Castell et al. (78).

CARBOHYDRATES

Background

The role of carbohydrate as an ergogenic aid for performance has long been recognised. The recommended daily carbohydrate intake for athletes who train for one to three hours each

day is 6–10 g/kg body mass increasing to 8–12 g/kg body mass for athletes training more than four hours each day, with additional intake of 30-60 g/h during exercise lasting for 1 hour or more (384). This guidance is principally aimed at restoring muscle and liver glycogen stores before exercise and maintaining blood glucose levels during exercise to ensure sufficient glucose availability for skeletal muscle contraction. However, carbohydrate availability also has the potential to limit the degree of exercise-induced immune dysfunction through direct or indirect actions. Directly, glucose acts as a fuel substrate for immune cells (12) therefore it could be argued that post-exercise hypoglycaemia could endanger immune cell function. However, the significance of this alone is questionable given that immune cells do not rely solely on glucose for energy. Conversely, since both catecholamines (adrenaline, noradrenaline) and cortisol are known to have potent modulatory effects on immune function (208) increasing carbohydrate availability may more likely act indirectly by reducing the stress hormone response to the exercise, thereby limiting exercise-induced immune impairments.

Consensus: carbohydrates, exercise and immune function

Dietary carbohydrates

Performing exercise at around 70% VO_2max for at least 1 hour following several days on very low carbohydrate diets (typically less than 10% of dietary energy intake from carbohydrate) is associated with a greater adrenaline and cortisol response, higher circulating neutrophil counts and modest depressions in circulating lymphocyte counts. These effects are diminished when exercising following a high (typically more than 70% of energy intake) carbohydrate diet (36,259). High carbohydrate diets prior to exercise are also associated with a blunted cytokine response (e.g. IL-6, IL-10 and IL-1ra) (37), thought to be related to a reduced need for IL-6 to exert its glucoregulatory actions (309).

In contrast, increasing dietary carbohydrate does not appear to exert any beneficial effects on either resting or post-exercise immune cell functions. A high or a low carbohydrate diet for several days was associated with similar levels of bacterially-stimulated neutrophil degranulation and mitogen-stimulated lymphocyte proliferation before exercise, and resulted in a similar magnitude of impairment post-exercise (36,259).

Carbohydrate supplementation during exercise

Given the established association between cortisol and immune cell function, nutritional measures that attenuate exercise-induced elevations in plasma cortisol have been hypothesized to be effective in minimizing post-exercise immune impairments. Specifically, carbohydrate (compared with placebo) ingestion during exercise is suggested to limit exercise-induced falls in immune function by maintaining plasma glucose levels, thereby blunting the plasma cortisol response. While evidence from the literature largely supports this, there is evidence to suggest that the beneficial effects of consuming carbohydrate during exercise can also occur in the absence of any effect on plasma cortisol levels (35,153). Consuming around 60 g/h of carbohydrate during prolonged exercise attenuates the rise in plasma cytokines (270), attenuates the trafficking of most leucocyte subsets, apart from NK cells (174,285,289), prevents the exercise-induced fall in bacterially-stimulated neutrophil degranulation (38) and increases neutrophil respiratory burst activity (351). In addition, consuming carbohydrate (compared with placebo) during prolonged exercise prevents the decrease in both number and percentage of anti-viral Type 1 helper T cells and the suppression of interferon gamma (IFN-γ) production from these cells (224). Consuming carbohydrate during exercise also diminishes typical post-exercise decreases in T lymphocyte proliferation following mitogen or antigen (influenza) stimulation (34,174), an effect that was still evident 24 hours later (34). This may be partially related to lower T cell apoptosis within stimulated cell cultures when carbohydrate is consumed during exercise (153). Migration of immune cells to infected tissue is crucial to host defence, and carbohydrate ingestion (60 g/h) during prolonged exercise has been shown to attenuate post-exercise falls in T-lymphocyte migration into human rhinovirus-infected airway epithelial tissue (35).

Although carbohydrate feeding during exercise appears to be effective in minimizing some of the immune perturbations associated with prolonged strenuous exercise, it has minimal effect on salivary secretory immunoglobulin A (SIgA) secretion (32,284) or NK cell cytotoxic activity (285) but may increase NK cell responsiveness to IL-2 (254). Consuming carbohydrate seems less effective at minimizing more modest alterations in immune function during intermittent exercise with regular rest intervals (288), resistance exercise (279) and exercise to fatigue (33). Furthermore, consuming more than 60 g carbohydrate per hour has negligible additional benefit (224,350,351) most likely because the maximum rate of exogenous carbohydrate oxidation is around 1 g/min (i.e. 60 g/h; (400)). Finally, carbohydrate supplementation does not influence the decrease in *in vivo* immunity to a novel antigen seen after 2 hours of moderate intensity exercise in non-fasted runners (102).

There is insufficient evidence to date to support any beneficial effect of carbohydrate ingestion on symptoms of upper respiratory illness; one study of 93 runners who consumed placebo or carbohydrate during a marathon reported that, of the sixteen runners who reported illness in the fifteen days after the race, ten had consumed placebo and six had consumed carbohydrate (284).

Conclusion

Carbohydrate ingestion is a partial countermeasure against exercise-induced immune impairment and is more effective when consumed as a supplement during exercise than by increasing dietary content of carbohydrate on a routine basis. However, evidence that carbohydrate ingestion reduces the incidence of URTI after prolonged exercise is currently lacking.

FATTY ACIDS AS IMMUNOMODULATORS

Background

Fatty acids are a major component of most human diets and most fatty acids can be synthesised endogenously in the human body (54). Individual fatty acids are distinguished by

the length of their hydrocarbon chain, and by the absence, presence, number and configuration (*cis* or *trans*) of double bonds within that chain. Saturated and monounsaturated fatty acids can be synthesised *de novo* from precursors such as glucose. The simplest polyunsaturated fatty acids (PUFAs), linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3), cannot be synthesised in humans, but are synthesised in plants. Humans can metabolise these two essential fatty acids further, inserting additional double bonds (desaturation) and extending the hydrocarbon chain (elongation). Through these processes, linoleic acid can be converted to arachidonic acid (20:4n-6) and alpha-linolenic acid to eicosapentaenoic acid (EPA; 20:5n-3). Further metabolism to longer chain, more unsaturated derivatives is possible (e.g., of EPA to docosahexaenoic acid (DHA; 22:6n-3)).

The principal roles of fatty acids are as energy sources and membrane constituents (54). Certain fatty acids have additional, specific roles, such as serving as precursors for the synthesis of bioactive lipid mediators (e.g. prostaglandins), and influencing membrane and intracellular signalling processes, the activation of transcription factors and gene expression (62). Through these different actions, fatty acids are able to influence cellular functions and thus physiological responses, including immune and inflammatory responses (59).

Consensus

There is evidence from *in vitro*, animal and epidemiological studies that several saturated fatty acids promote inflammatory processes (334). Lipid mediators, including prostaglandins and leukotrienes, produced from the omega-6 (n-6) PUFA arachidonic acid are intimately involved in inflammation and many widely used anti-inflammatory drugs target arachidonic acid metabolism (106). Several of the mediators produced from arachidonic acid also suppress cell-mediated immune responses by targeting antigen-presenting cell and helper T-cell activities, acting in part via regulatory T-cells (106). Arachidonic acid is consumed in the diet from meat, eggs and organs such as liver, or it can be synthesised from the plant essential fatty acid linoleic acid. Thus, there is a widely held view that common n-6 PUFAs of both animal and plant origin are pro-inflammatory and immunodepressive. However, strong evidence that variations in dietary intake of linoleic acid do affect inflammation is lacking (197). Older research showed that γ-linolenic acid (18:3n-6) and its derivative dihomo-γ-linolenic acid (20:3n-6), which are both metabolic intermediates between linoleic and arachidonic acids, exert anti-inflammatory effects (360). There is limited exploration of the influence of saturated or n-6 PUFAs on immune function or inflammation in the context of exercise.

Oily fish and fish oil supplements contain the long-chain omega-3 (n-3) PUFAs EPA and DHA (60). EPA and DHA are also found in some algal oils and in krill oil. In each of these sources both the absolute amounts of EPA and DHA and their ratio can vary widely. There is substantial evidence from *in vitro*, animal, epidemiological and human intervention studies that the combination of EPA and DHA exerts anti-inflammatory actions (58,61) and may enhance cell-mediated immune function (59). The effects observed are dose-dependent and may require an intake of >2 g per day the combination of EPA and DHA (58,59,61). Both EPA and DHA can independently exert anti-inflammatory effects (5), and they have been shown to counter the effects of classic inflammatory stimuli like endotoxin as well as saturated fatty acids and n-6 PUFAs (61). EPA and DHA are readily incorporated into cell membranes, partly replacing arachidonic acid. Thus, they result in decreased production of pro-inflammatory and immunosuppressive omega-6-derived lipid mediators (61). In contrast, the analogous mediators produced from EPA are often only weakly bioactive (61). Importantly, both EPA and DHA are substrates for the biosynthesis of potent mediators which resolve inflammation and enhance immune function: these are termed resolvins, protectins and maresins (19). EPA and DHA act through several other mechanisms to decrease inflammatory responses of neutrophils, macrophages and endothelial cells. These mechanisms include reducing activation of the pro-inflammatory transcription factor NFκB and activation of peroxisome proliferator activated receptor γ (61). Within antigen-presenting cells, T-cells and B-cells EPA and DHA act by regulating key signalling events within the cell membrane (184).

Effects of the combination of EPA and DHA, usually as fish oil, or of DHA alone, on inflammation and immune function have been explored in a number of studies involving exercise protocols in both athletes and non-athletes. Several studies report that supplementation with EPA and DHA decreases the degree of muscle soreness induced by a bout of exercise in untrained individuals (201,382,383), although not all studies saw this (151). This effect also occurred with DHA alone at a supplemental intake of 3 g/day (91). Furthermore, in untrained individuals, EPA and DHA have been reported to diminish the exercise-induced elevation in pro-inflammatory cytokines including TNF-α and IL-6 (383). Once again this effect was also seen with DHA alone (108). Thus, the majority of studies suggest that omega-3 PUFAs decrease the inflammatory response induced by exercise in untrained individuals and that this translates to less muscle damage and soreness. In contrast, Gray et al. (2012; (152)) and Da Boit et al. (2015; (99)) both observed no effect of EPA and DHA on the plasma IL-6 response to an exercise bout, although the production of IL-2 by mitogen-stimulated blood mononuclear cells and natural killer cell activity were both enhanced post-exercise with prior omega-3 PUFA treatment in both of these studies. These observations suggest that omega-3 PUFAs might enhance aspects of cell-mediated and innate immunity post-exercise.

Andrade et al. (2007; (10)) reported that elite swimmers who took EPA and DHA for 45 days showed increased production of IL-2 and increased T-cell proliferation when peripheral blood mononuclear cells were stimulated with the mitogen phytohaemagglutinin, although IFN-γ production was decreased, making the findings difficult to interpret. There was no effect of EPA and DHA over 6 weeks on salivary IgA concentration prior to or after an exercise bout in trained cyclists (287). There was also no effect on pre-or post-exercise blood leucocyte numbers or the concentrations of C-reactive protein, IL-1β, IL-6 or IL-8 (287). A study of high dose n-3 PUFAs (3 g/day EPA plus 1.8 g/day DHA) given for 6 weeks to endurance trained males reported no effects on marathon-induced changes in blood leucocyte numbers or blood concentrations of TNF-α, IL-1ra, IL-6 or transforming

growth factor beta (388). Likewise, high dose EPA and DHA (2.2 g/day of each) for 6 weeks had no effect on treadmill exercise induced changes in several blood inflammatory markers in trained males (43). A small study in wheelchair basketball athletes found that EPA and DHA prevented the elevation in plasma IL-6, but not other pro-inflammatory cytokines, induced by an exercise bout, and prevented damage to neutrophils (244). Capo et al. (66) reported few effects of EPA and DHA on several plasma cytokines in elite soccer players undergoing an exercise bout, although there was a weaker TNF-α and IL-6 response of peripheral blood mononuclear cells to endotoxin in the n-3 group PUFA after exercise. This was linked to a lower exercise-induced upregulation of toll-like receptor 4 in the n-3 group (66). Other studies present results from studies of n-3 PUFAs on immune outcomes in athletes that are difficult to interpret (105,346).

Controversies and Future Directions

There is limited exploration of the influence of saturated or n-6 PUFAs on immune function or inflammation in the context of exercise. There are a number of studies of the long chain n-3 PUFAs EPA and DHA, usually in combination, on immune function or inflammation in the context of exercise in both untrained and trained individuals. These studies have used moderate (<1.0 g/day) to high (4 g/day) doses of EPA plus DHA for a period of one week to several months; some publications are not sufficiently clear about the n-3 dose used and many studies have involved a small number of subjects. Few studies have used the same design and the immune and inflammatory outcomes reported are highly variable between studies, although several report measurement of the same common plasma inflammatory cytokines. This makes it difficult to draw firm conclusions. However, EPA and DHA appear to decrease exercise-induced inflammation and muscle damage and soreness in untrained individuals. It is not yet clear whether EPA and DHA affect inflammation or immune function in trained individuals, although some studies suggest they might. Larger studies of duration of several weeks to months are recommended to explore better the effects of omega-3 PUFAs, and other fatty acids, on inflammation and immune function in trained individuals. Immune and inflammatory outcomes to be measured should be more carefully considered (2,3).

AMINO ACIDS

Amino acids are the building blocks for protein. Twenty amino acids build up the body proteins, nine of these are considered essential, that is, they have to be supplied through the diet; the remaining, non-essential, amino acids can be synthesized by the body. Exercise increases the oxidation of amino acids, and both synthesis and degradation of muscle protein are increased after exercise (312). The rate of synthesis can be elevated for up to 72 hours after exercise (258). Recent consensus indicates that amino acid requirements are increased with regular exercise training, suggesting that protein intakes as high as ~150-200% of current recommendations might be necessary for these individuals (313).

Several amino acids also have other important roles, for example serving as substrates in the synthesis of neurotransmitters, stimulating protein synthesis or improving immune function. A list of amino acids involved in immunology and their roles can be found in a comprehensive review by Li et al. (2007; (230)). The most studied amino acids in exercise immunology are Gln, BCAA, alanine and arginine. Gln and BCAA are the most studied in terms of supplementation before and after exercise.

Branched chain amino acids

During prolonged, fatiguing exercise the branched chain amino acids (BCAA) (leucine, isoleucine and valine) are taken up by the working muscle and their plasma concentration decreases (41,397). The BCAA are oxidized to provide energy but, more importantly, the metabolism of BCAA produces nitrogen for Gln synthesis.

Intake of BCAA rapidly increases their plasma and muscle concentration (42,154,260), and it is suggested that this will increase the production of Gln. A fall in the plasma concentration of Gln (p[Gln]) has been observed during/after sustained exercise (104,332), and has been proposed to be linked with exercise-induced immunodepression ((77,336); see Glutamine chapter). BCAA intake could therefore indirectly influence the immune response. However, despite the elevated levels of BCAA in plasma and muscle after intake of these amino acids, the release of Gln from the exercising muscle remains unchanged unless large amounts of BCAA are ingested (42,236,237). In contrast to these findings, chronic supplementation with BCAA to athletes was able to prevent the decrease in p[Gln] and immunodepression following a triathlon or a 30 km race (21). Furthermore, ten weeks of BCAA supplementation to trained cyclists prevented the increase in neutrophil number in trained cyclists which was observed without supplementation (207).

A direct effect of BCAA on cells of the immune system may also be conceivable since these amino acids, in particularly leucine, may stimulate protein synthesis and activate cytokine and antibody production through a direct effect on mTOR signalling (see (230)). However, there is currently no evidence for such an effect, and available data indicate that BCAA are required to maintain the high rate of protein synthesis in these cells rather than to stimulate the immune function (see (57,97)).

Consensus

There are some indications that BCAA intake can reduce exercise-induced immunodepression. However, there is currently not enough data from controlled studies to recommend BCAA ingestion in combination with exercise to enhance immune function.

Glutamine

Background

Glutamine is the most abundant amino acid in the body and was originally classified as a non-essential amino acid (340). However, since the 1990s there has been increasing evidence that Gln becomes “conditionally essential” in specific conditions of stress (73,220).

Gln is synthesized, stored and released predominantly by skeletal muscle and, to a lesser extent, by adipocytes, liver and lung. It is taken up by intestinal cells, such as enterocytes and colonocytes, by the kidney, liver and immune cells such as lymphocytes, macrophages and neutrophils. It has been suggested that Gln supplementation might improve the digestive and defence mechanisms of the intestine (100,410).

Gln is required by rapidly-dividing cells (213), providing nitrogen for purine and pyrimidine nucleotide synthesis, enabling synthesis of new DNA and RNA, for mRNA synthesis and DNA repair. Ardawi and Newsholme (1985; (12,13)) observed a high Gln utilisation by human lymphocytes at rest. Subsequent *in vitro* work (303) showed that when Gln was reduced in culture medium a decrease occurred in the proliferative ability of human lymphocytes. Gln and BCAA (see Amino acids section) are the most studied amino acids in terms of supplementation before and after exercise.

The p[Gln] is increased in athletes after short-term exercise (316). However, after prolonged, exhaustive exercise such as a marathon, the p[Gln] can be decreased by 20-25% (77,104). Similar decreases have been observed after repeated bouts of prolonged exercise (336). In two studies at moderate altitude (athletes, in summer) and high altitude (military personnel, in winter) a significant decrease in p[Gln] occurred after intensive training and coincided with a high incidence of URTI (16,79).

The post-exercise decrease in p[Gln] is often concomitant with a decrease in circulating lymphocyte numbers which transiently increase initially as part of the well-known leucocytosis observed after exhaustive exercise. Immune cell function is also decreased at this stage, for example, in both lymphocytes and NK cells. Rohde et al. (1996; (336)) observed a marked decrease in p[Gln] in triathletes at 2 hours after prolonged exercise, paralleled by changes in lymphokine activated killer (LAK) cell activities. A decrease in p[Gln] in marathon runners coincided with increases in acute phase markers such as the cytokine IL-6 and complement C5a, as well as an increased incidence of self-reported URTI (76,77).

There is some evidence that Gln, or a Gln precursor (BCAA), can lessen the incidence of exercise-induced URTI after marathon running (21,77). However, several studies, mostly laboratory-based, have shown no effect of maintaining a normal or high p[Gln] on various aspects of immune function. These included: LAK cell activity, lymphocyte numbers, some leucocyte subsets, salivary IgA, CD3 T-cell receptors, NK cells, leucocytosis, plasma elastase release from lipopolysaccharide (LPS)-stimulated neutrophils (216,335,337,403); CD8, CD4 with/without CD28 & 9 surface receptors (215), although the latter study also showed less neutrocytosis in the Gln group than the placebo group.

In a recent study Caris et al. (2014; (67)) observed a positive effect of both Gln and carbohydrate in modulating the Th1/Th2 (helper cells) balance after exercise. The post-exercise ratio of $CD4^+$ helper/ $CD8^+$ cytotoxic/suppressor cells has been seen to be higher in athletes provided with Gln rather than placebo after both marathon running (75) and heavy load training (373).

It has been suggested that muscle Gln is not markedly decreased as a result of exercise, although Rennie et al. (1981; (332)) did see a decrease in muscle Gln in their study, which also produced a biphasic response of p[Gln] to 3.75 hours of exercise. The decrease is markedly less than the pathologically low Gln concentrations observed in the muscle of critically ill patients (341). Current opinion considers that, in general, the body has sufficient stores of Gln to replenish post-exercise plasma reductions readily (see (139)). The time frame for this is not known. Interestingly, Hiscock et al. (2002; (180)) measured the intracellular content of Gln in peripheral blood mononucleocytes (PBMC), and found good availability of Gln for the cells after exercise.

The presence of glutaminase, the major degradation enzyme of Gln, was established in human neutrophils by Castell et al. (2004; (74)). There appears to be a link between production of the major neutrophil chemoattractant, IL-8, and Gln. In i*n vitro* studies the provision of Gln results in a decrease in IL-8 production in athletes (73), and in clinical studies in patients with acute pancreatitis (23). Provision of exogenous Gln might therefore lead to a decrease in the requirement for IL-8 secretion to attract more neutrophils to the site of tissue damage, though this is speculative.

IL-6 is probably the most studied cytokine (myokine) in exercise immunology. The plasma concentration of IL-6 increases markedly after strenuous and prolonged exercise and this increase was further enhanced after Gln supplementation (181). This might prove beneficial if, as has been suggested, IL-6 acts as an anti-inflammatory cytokine in exercise (311).

In regard to leucocytosis after endurance exercise, Fehrenbach et al. (1999; (126)) described a possible protective effect of heat-shock protein (HSP) in athletes. There is substantial evidence that Gln is important for HSP generation in both *in vitro* and *in vivo* studies (200,423,426). Zuhl et al., 2014 (433) observed anti-inflammatory effects of Gln via HSP70 on intestinal permeability and peripheral blood mononuclear cells. Raizel et al. (2016; (327)) recently showed that treating rats with oral free L-Gln (with L-alanine or as a dipeptide) induced cytoprotective effects via HSP70 after resistance exercise. HSP facilitates neutrophil activity (179,296): given the presence of glutaminase on the secretory granules of human neutrophils (74), the effect of Gln on the heat shock response might induce changes in neutrophil function.

Consensus

Despite a good rationale for Gln supplementation based on sound biochemical investigation, laboratory-based exercise studies have proved disappointing in terms of providing any direct enhancement of immune function due to Gln feeding (see Controversies). Recently, it has been suggested that there is sufficient Gln availability in body stores to combat post-exercise decreases in immune function after endurance events. Nevertheless, a decrease in p[Gln] may act as a marker for immunodepression and increased incidence of minor illnesses. Thus, a marked decrease in p[Gln] may indicate

decreased immunocompetence, in particular in the individual who is vulnerable to opportunistic infections. There are some indications that provision of Gln or a Gln precursor can lessen the incidence of exercise-induced URTI.

Controversies

Since p[Gln] decreases by approximately 20-25% after prolonged, exhaustive exercise, given its role in some key immune cells, this might be expected to have ramifications for immune function in athletes. There was a sound biochemical and clinical rationale for thinking that Gln provision might be a simple panacea for minor illnesses and for exercise-induced immunodepression. Despite the evidence that Gln or a Gln precursor can lessen the incidence of exercise-induced URTI, several laboratory-based studies have shown no effect of maintaining a normal or high p[Gln] on some specific aspects of immune function.

Future Directions

Data on the effects of supplementation with Gln or Gln precursors on neutrophil function in exercise have become increasingly interesting, and further investigation in humans should prove to be useful. Gln has a role in generating heat shock protein: this might have a protective effect on immunodepression in exercise, and more studies are required. There may also be other aspects of immune function as yet unstudied, which might respond more effectively to the provision of Gln before or after prolonged, exhaustive exercise.

MINERALS

Background

Several minerals are known to exert modulatory effects on immune function, including Zn, Mg, Fe, Se, and Mn. With the exception of Zn and Fe, isolated deficiencies are rare. Regarding exercise, requirements for some of these minerals are certainly higher in athletes compared with sedentary people. On the one hand, exercise has a pronounced effect on mineral metabolism; on the other hand, exercise increases losses in sweat and urine. However, excess intakes of some minerals are known to impair immune function. Earlier reviews have discussed mineral supplementation comprehensively (63,138). The present consensus statement considers supplementation of five specific minerals (Zn, Mg, Fe, Se and Mn) in relation to exercise.

Consensus

Zinc

The essential trace element Zn is an important co-factor of several enzymes and transcription factors and thereby involved in various physiological processes during growth, metabolism, and development. Studies with hereditary diseases of Zn deficiencies such as acrodermatitis enteropathica have demonstrated the importance of proper Zn levels for immune function of both adaptive and non-adaptive systems (187). Severe Zn deficiency in these patients is accompanied by several symptoms including enhanced susceptibility to infections. But even mild Zn deficiency occurring in populations at risk such as elderly people or vegetarians may result in impairment of NK cell lytic activity and T cell mediated functions (319). Intracellular Zn levels in T cells seem to be highly regulated and involved during T cell activation. In macrophages Zn seems to play a part in important anti-inflammatory roles by inhibiting NF-κB signalling. Beside its action on immune cells Zn seems to have direct anti-viral properties via Intercellular Adhesion Molecule (ICAM)-1 receptors on respiratory epithelial cells of the nasal epithelium (186).

The RDA of Zn for men and women in the US is 11 and 8 mg, respectively; in the EU a gender independent value of 10 mg is given. Zn can be found in a wide variety of foods like certain types of sea food such as oysters, crabs and lobsters, red meat, poultry, beans, nuts and whole grains. In contrast, bioavailabilty of Zn is impaired by phytates which are present in whole-grain breads, cereals, and legumes, and by Fe supplementation.

There is considerable mobilization of Zn during exercise into the blood, which is re-distributed soon after termination of exercise. Nevertheless losses of Zn via sweat and urine, in addition to reduced dietary intake, have been identified as major risk factors for Zn deficiency in athletes. Therefore a number of studies reported Zn deficiency (serum levels < 70 µg/dl) in elite athletes, especially in endurance athletes (257). However, the impact of these alterations on athletes' immune system/function remains to be shown. Therefore regular supplementation of Zn cannot be recommended. Nevertheless, there is some evidence from general population studies that Zn supplementation might be effective in the prevention and therapy of the common cold, which represents the major disease form of athletes during transient immunodepression in the early post-exercise period. A recent study presented weak evidence for Zn in the prevention of the common cold in children (6). In addition, recent meta-analysis including 17 trials and a total of 2121 participants presented moderate evidence that oral Zn formulations may shorten the duration of symptoms of the common cold (354). It has been suggested that supplementation should start within 24 hours of the onset of symptoms (367). Based on these studies, a transient supplementation during periods of intensive exercise bouts together with psychological stress such as during competition might be beneficial, especially if a history of recurrent infections exists. Side effects of Zn supplementation include bad taste and nausea.

Magnesium

Mg is an essential biological element which is predominantly located in bones (approx. 52%), in muscle cells (28%), and soft tissue (19%). Serum and red blood cells contain only 0.3% and 0.5%, respectively. In general, Mg is involved as an important regulator in three main physiological processes; 1) enzyme activation, e.g. during energy metabolism, 2) stabilizing membrane function and integrity, 3) cell signalling, e.g. as a natural antagonist of intracellular calcium signals (263). With respect to the function of the immune system Mg seems to be involved in the following steps: cofactor for immunoglobulin synthesis, immune cell adherence, antibody-dependent cytolysis, activation of macrophages. Moreover, Mg deficiency is associated with clinical signs of inflammation, such as immune cell activation and enhanced levels of circulating inflammatory mediators (222).

The concentration of total serum Mg is approximately 0.75-1.1 mmol/l, which is, however, a rather poor indicator of the body's Mg status. Serum acts as a transit pathway between electrolyte uptake and excretion, bone stores and actively metabolising tissues. These processes are affected by a number of hormones such as parathyroid hormone, calcitonin, vitamin D, insulin, glucagon, antidiuretic hormone, aldosterone and sex steroids.

Exercise-induced alterations of serum Mg seem to depend on exercise intensity and duration. After short-term, high-intensity exercise the majority of studies indicated an increase of extracellular Mg; however, after prolonged submaximal exercise most studies reported a hypomagnesaemia (56). It seems unlikely that sweat Mg losses and/or enhanced renal Mg excretion alone account for this decrease in serum. Some authors suggested therefore that, during prolonged exercise, a shift of Mg into the cellular compartment occurs. Longitudinal and cross-sectional studies demonstrated that intensive training periods may be followed by Mg depletion and that athletes are prone to Mg deficiency (348).

Therefore it can be speculated that the exercise-associated changes in immune function especially in the early post-exercise period might be aggravated in Mg-deficient athletes (222). In contrast, it has been demonstrated that Mg supplementation did not prevent exercise-induced alterations of immune parameters in athletes with balanced Mg status (262). Therefore, Mg supplementation can be recommended only after diagnosis of Mg deficiency which relies on both clinical symptoms and laboratory diagnosis (serum Mg < 0.75 mmol/l is considered to be a useful measurement for severe deficiency). Important food sources of Mg are vegetables, fish, nuts, and whole grains. Mg formulations include both inorganic and organic compounds of which the latter seemed to have a better bioavailability.

Iron

Fe is an essential nutrient which is primarily used as a cofactor for enzymes in the mitochondrial respiratory chain, in the citric acid cycle and during DNA synthesis, as well as being the central molecule for binding and transport of oxygen by haemoglobin and myoglobin (414).

For immunity, Fe is important for lymphocyte proliferation and differentiation while it interferes with cell mediated immune effector pathways and cytokine activities (356,414). Furthermore, Fe exerts multiple effects on macrophage polarization and functionality (269).

Changes in Fe status can thus affect the immune response in multiple ways, particularly in the context of infection (82). The RDA is 18 and 8 mg for women and men respectively. Sources of Fe are flesh foods, vegetables and grains. The haem Fe, found in meat products, is best absorbed. In general, male athletes tend to consume at least the RDA for Fe, but female athletes tend to consume somewhat less (166). If this under-supply is combined with heavy Fe loss by menstruation, haemolysis, gastrointestinal bleeding, inflammatory status by heavy physical activity or loss by sweat, Fe balance may be compromised (235,253). Accordingly, Fe deficiencies have been reported mainly in women competing in running, field hockey, cross country skiing, basketball and others (253). In this case, the use of Fe fortified foods and Fe supplements may be considered (51). Therefore, Fe supplementation in combination with vitamin C should be recommended for athletes with Fe deficiency anaemia and monitored carefully for prophylaxis. During infection the supplementation of some minerals like Fe is not recommended because it is suggested that pathogenic microorganisms might benefit (107). However, an immunological effect of Fe supplementation in the context of exercise has not been shown so far.

Selenium and Manganese

Se status may affect the function of cells of both adaptive and innate immunity. Currently, the recommended amounts for adequate Se intake of adults range between 25 and 100 µg/day, with an average of 60 µg/day for men and 53 µg/day for women (328,376). Neither Se deficiencies nor immunological effects of supplementation in athletes have been described yet. For Mn, daily intake through dietary sources provides the necessary amount required for several key physiological processes, including antioxidant defence, energy metabolism, immune function and others. During exercise, Mn might play a role as an antioxidant since a superoxide dismutase in the mitochondrial matrix functions with Mn. There is no evidence for neither deficiency nor supplementation for Se nor Mn in athletes, thus both minerals cannot be classified as immunonutrition during exercise (81). An overview about these minerals, their immune related functions, symptoms of deficiency, deficiencies in sports and recommendations for supplementation is given in Table 1.

Future directions

While there is evidence that regular exercise training of high volume and intensity may be accompanied by deficiencies of certain minerals such as Zn, Mg and Fe, the impact of these alterations on the athlete's immune function needs to be demonstrated. *In vitro* experiments could demonstrate the involvement of these ions in certain immune processes. But it remains to be shown whether these deficiencies are able to aggravate exercise-induced immune responses.

ANTIOXIDANTS

Background

Free radicals are reactive molecules with unpaired electron(s) (158). High levels of free radicals damage cellular components. Antioxidants are chemical compounds and enzymes that exist as a natural means of quenching free radical overproduction. However, moderate levels of radicals and other oxidants are central to the control of gene expression, cell signalling pathway regulation, and physiological modulation of skeletal muscle force production (318). In the context of inflammation in health and disease, genomic, cellular, and physiological outcomes are regulated by fluctuations between free radical species and their antioxidant counterparts. In this section, we outline oxidants (mainly ROS and reactive nitrogen species (RNS)) and antioxidants (with a focus on dietary sources of antioxidants), their effects on exercise, and the interface with the immune system (329).

Table 1: Overview about specific minerals, their immune related functions, symptoms of deficiency, deficiencies in sports and recommendations for supplementation.

	Immune related functions	Deficiency signs or symptoms (general)	Deficiencies in sports	Supplementation
Zinc	Adaptive and non-adaptive immune responses	Enhanced susceptibility to infections	Zn deficiency has been described in elite athletes, especially in endurance athletes	Regular and continuous supplementation cannot be recommended; short-term Zn supplementation might be effective in common cold therapy; transient supplementation during intensive physical/ psychological stress can be considered especially if there is a history of recurrent infections
Magnesium	Cofactor for immunoglobulin synthesis, immune cell adherence, antibody-dependent cytolysis, activation of macrophages	Enhanced neuromuscular excitability, muscle cramps, anxiety, and clinical signs of inflammation, such as immune cell activity, increased circulating inflammatory mediators	Endurance athletes; sports using protective clothing, which increases Mg losses via sweat	Mg supplementation in athletes with balanced Mg status cannot be recommended. Supplementation can be recommended only after diagnosis of Mg deficiency.
Iron	Haemoglobin/ myoglobin synthesis, energy metabolism Lymphocyte proliferation and differentiation macrophage polarization and functionality	Anaemia, cognitive impairment, weakness, immune abnormalities	Mainly women competing in running, field hockey, cross country skiing, basketball	Recommended for athletes with iron deficiency anaemia and monitored carefully for prophylaxis, no data about immunological effects
Selenium	Se acts mainly through selenoproteins, e.g. antioxidant selenoenzymes such as glutathione peroxidases (GPxs) and thioredoxin reductases (TrxRs)	Increased risk of Keshan disease and Kashin-Beck disease (both often occur in conjunction with iodine deficiency or environmental toxins)	Not described	Not described
Manganese	Manganese superoxide dismutase might play a role as an antioxidant	Not described	Not described	Not described

Relevant to immune system interactions, superoxide is one of the strongest cellular oxidants, but it is quickly dismutated to hydrogen peroxide by the enzyme superoxide dismutase. Hydrogen peroxide is a more stable, non-free radical ROS that is permeable to cellular membranes. Despite being a weak oxidizing agent, high local concentrations of hydrogen peroxide are cytotoxic. Toxicity is typically associated with oxidizing chain reactions promoted by Fe (Fenton reaction) centered molecules which produce hydroxyl radicals. Hydroxyl radicals are strong oxidants, highly reactive and, when concentrated, can be the most damaging ROS in biological systems. Central to inflammation, hypochlorite is another well-known ROS product (329). Hypochlorite is formed by the action of the oxidative enzyme myeloperoxidase utilizing hydrogen peroxide. Hypochlorite is produced by neutrophils and macrophages (233,432) and, independent of pathogen defence, can oxidize circulating cholesterol and other humoral factors with deleterious consequences. Moreover, this oxidant readily forms hypochlorous acid, subsequently crossing cell membranes and damaging essentially all cellular constituents with negative effects (71).

Nitric oxide is held to be the main RNS in inflammatory processes and is synthesized enzymatically (nitrogen oxide synthase isoforms) from the amino acid L-arginine. Nitric oxide is a weak reducing agent, but can react with superoxide to produce peroxynitrite. As with ROS, RNS promote health or disease within the context of a dose, duration and the local biochemical environment. In certain scenarios peroxynitrite is a strong oxidizing agent that depletes thiol groups, and damages DNA and proteins (234).

Consensus

Undeniably, participation in acute exercise is associated with a transient production in the ROS/RNS (317). Moreover, while the source of ROS/RNS production is largely thought to be generated within the contracting skeletal muscle (331) there is ample evidence that the immune system is also responsible for exercise-generated radical species (325).

The involvement of the immune system in ROS and RNS production

The rate of oxygen consumption by phagocytes (e.g., neutrophils, eosinophils and mononuclear phagocytes) increases when exposed to certain stimuli (e.g., pathogens, pollutants, etc.). When this occurs, the phagocytes produce high levels of superoxide and collectively these events are known as the "respiratory burst" (14). The purpose of this phenomenon is to

generate powerful microbiocidal agents by the internal defence arm of the immune system. Specifically, macrophages produce NO which plays a critical role in redox-related functions of the immune response (422).

Interaction of exercise and antioxidant content (potential) of the immune system

It is well established that prolonged, high intensity training and competition can result in acute immune impairment in athletes and usually manifests as an increased susceptibility to minor illnesses, particularly URTI (see Section 12). Essential to this understanding is the fact that acute high intensity exercise is associated with upregulation of endogenous antioxidant enzyme transcripts (129). This finding is important and suggestive of the fact that, as with muscle level adaptations, the immune system is resilient and ultimately adapts beneficially to exercise.

Controversies

Advocates of antioxidant supplementation argue that exercise-induced oxidant production cannot be adequately quenched without dietary intervention. While this logic may seem reasonable at first, several arguments contradict the notion that athletes and recreational exercisers require dietary antioxidant supplementation. For example, dietary antioxidant consumption has been proposed to reduce the risk of respiratory illness, but conclusive data to support or oppose this statement are not available (121). Furthermore, links between exercise-induced oxidative stress and immune dysfunction (275), and the postulated benefits of dietary antioxidant supplementation in preventing immune dysfunction during exercise, are not substantiated empirically (276).

In addition, to counteract the proposed need for supplemental antioxidants, there is no conclusive evidence that exercise-induced ROS production is detrimental to human health. By contrast, the fact that exercise elicits both oxidative stress and numerous adaptive health and athletic performance benefits is paradoxical to the idea that supplemental antioxidants are needed. Moreover, regular exercise training promotes fortification of endogenous enzymatic and non-enzymatic antioxidants, a fact that extends to circulating immune cells from exercised individuals (129). The adaptive increase in endogenous antioxidants does not fully quench the ROS/RNS generated during the exercise, but is clearly sufficient to protect against deleterious outcomes due to exercise-induced oxidative stress (194).

According to scientific consensus, therefore, athletes who consume an appropriate energy intake from nutrient-dense foods do not need antioxidant supplementation. Moreover, there is no evidence that exercise in extreme environments necessitates antioxidant supplementation (324). In contrast, one feasible circumstance in which supplemental dietary antioxidants may be warranted is in individual cases of nutrient deficiencies (e.g., antioxidant status below the normal range for good health). This latter instance is a rare exception to our broader understanding of exercise and oxidative stress: nevertheless it has been questioned scientifically by investigating the dietary practices of athletes (396). Importantly, emerging evidence indicates that antioxidant supplementation mitigates important exercise-induced adaptations which now appear to extend to the immune system.

Future directions

The debate for and against antioxidant supplementation in athletes and regular exercisers appears likely to continue despite comprehensive understanding of immunonutrition and exercise-induced oxidative stress. Antioxidant supplementation practices are often driven by business models and consumer biases that seek a convenient means to improve athletic performance, health, and longevity. Accordingly, there is a pressing need for additional research to demonstrate when, and in what context, antioxidant supplementation may be efficacious. Moreover, future work should be mindful of corporate biases and include points for consumer advocacy whenever possible. It is proposed that exercise and nutritional scientists should join with practitioners to educate athletes about the current scientific understanding regarding antioxidant supplements and exercise-induced oxidative stress and inflammation. Education efforts should be strategic and ever mindful of consumer demand for pill-based solutions to complex problems like performance enhancement, a point that is of particular importance to exercise and immunonutrition.

PLANT-DERIVED IMMUNOMODULATORS: HERBAL SUPPLEMENTS

In this article "botanical supplements" ("herbal supplements") refers to single- or multi-organ plant extracts in tablet or liquid form containing a diverse array of phytochemicals, in contrast to "botanicals" ("herbals") which refers to isolated plant compounds or compound groups.

Background

Several plants are used by athletes as dietary/nutritional supplements (Table 2). Quantifying global rates of use is logistically problematical because athletes and researchers differ in their definition of "herbal supplement", and multicomponent preparations or foodstuffs may contain herbs unbeknownst to athletes. Usage surveys sometimes neglect herbal supplements or lump different supplements together as one group (123,211,359). Thus, use is likely to be underestimated.

Athletes consume herbal supplements for both health and performance reasons, and a given supplement often has more than one presumed use. Many herbal supplements consumed by athletes have purported immunomodulatory capacities (Table 2). Presumed immunomodulatory herbal supplements are diverse in terms of taxonomy, plant organs used, and bioactive compounds.

Consensus

Empirical evidence for the immunomodulatory capacities of herbal supplements is often incomplete, equivocal, and/or weak, whether the studies used athletes/non-athletes or exercise/non-exercise models. Ginseng and echinacea possibly serve as the best models for examining immunomodulatory herbal supplements in athletic contexts, because they have been more robustly researched, and bioavailability studies suggest these supplements' bioactive molecules can pass

Table 2: Herbal supplement use by 8424 athletes based on 27 published surveys. Data used for this table were gleaned from a subset of 27 athlete surveys identified by Knapik et al. (211) as containing references to specific herbal supplements (references 9-12, 15, 19, 23, 35, 49, 58, 67, 68, 80, 101, 121, 123, 128, 131, 132, 141, 144, 149, 151-153, 159, and 198 in (211)), but were analyzed differently here. * = Many herbal supplements were quantified only once and were not tabulated: alfalfa, chamomile, ciwujia, evening primrose, goldenseal, green tea, guarana, kava kava, kola nut, peppermint, tea tree oil, and yohimbe. Some surveys also noted "herbal supplements" (4 surveys; 0.9 ± 2.9%) or mixed herbal preparations (4 surveys; 3.5 ± 10.5%). † = "Yes" indicates at least some use among athletes as an immunomodulator, and "no" indicates the supplement is not consumed as an immunomodulator; designations do not connote whether the plant is primarily taken as an immunomodulator (e.g., echinacea) or only secondarily (e.g., ginseng), or efficacy. ‡ Two additional studies did not provide usage statistics. § One additional study did not provide usage statistics.

Supplement*	# surveys reporting use	Average % (± s.d.) of respondents using supplement (across all 27 surveys)	Purported immune-modulator?†
Ginseng	20‡	10.6 ± 17.6%	Yes
Echinacea	14	9.5 ± 18.0%	Yes
Garlic (+/- horseradish)	4§	3.1 ± 11.8%	Yes
Ginkgo	4§	0.9 ± 2.6%	Yes
Spirulina (blue-green algae)	4§	0.4 ± 1.4%	Yes
Ephedra	3	1.7 ± 5.8%	No
St. John's Wort	3	0.3 ± 1.1%	Yes
Flax, Flaxseed	2	0.2 ± 1.0%	No
Tribulus	2	0.1 ± 0%	No

through the gut into the bloodstream in physiologically relevant quantities.

Echinacea is primarily taken by athletes for prevention or treatment of upper respiratory tract infections such as colds or influenza. Recent reviews and meta-analyses from clinical trials with the general population concur that echinacea supplementation may lessen symptom severity or duration, but are equivocal in their assessment of its prevention capabilities (204,349). Results from athlete/exercise studies on echinacea are similar (Table 3) (27,155,353). Though all three studies used *E. purpurea*-based preparations, Table 3 epitomizes the problems in forming conclusions about echinacea supplements (or most herbal supplements for that matter; e.g., few studies, different populations, measurements, exercise interventions, and treatment interventions). One recent review that examined clinical studies, *ex vivo* studies (where blood samples were drawn pre- and post-exercise, but lymphocytes were stimulated *in vitro*), and *in vitro* studies concluded that echinacea supplements may stimulate both innate and adaptive immunity (the former more so) and that alk(yl)amides and caffeic acid derivatives are the likely bioactive molecules (358). In terms of ergogenic potential, echinacea supplementation did not improve endurance capacity or VO_2max in three studies (22,25,375) but did in one (417).

Ginseng is primarily taken by athletes as an ergogenic or adaptogenic aid, either as a standalone supplement or in multicomponent "energy drinks." Recent reviews have discounted its utility as an ergogenic aid (15) and further suggest that any benefits seen in energy drinks are likely attributable to caffeine or sugars and not ginseng (18). Ginsenosides are the presumed immunomodulatory constituents. A review of the immunomodulatory effects of ginseng supplements in athletes has been provided elsewhere (Table 2 in (358)). Though there are more *in vivo* studies on ginseng and its potential immunomodulatory effects than echinacea, outcomes were worryingly inconsistent across studies and often weak, likely owing to the diverse species, extract types, and dosing used. The two studies investigating IL-6 are a good example of this predicament. In one study (202), trained males consumed heat-treated P. *ginseng* supplements for seven days before two 45-minute treadmill runs and demonstrated reduced IL-6 compared to controls a couple hours post-exercise but not a day later. In a separate study (225), untrained male subjects consumed *P. pseudoginseng* for three days before 30 minutes of treadmill running at 60% VO_2max and demonstrated no difference in IL-6 levels post-exercise compared to controls.

Table 3. Representative studies concerning potential immunomodulatory effects of Echinacea supplementation in athletes. Abbreviations: RA = recreationally-active, TR = trained.

	Berg et al. 1998 (27)	Hall et al. 2007 (155)	Schoop et al. 2006 (353)
Population	42 TR ♂	32 RA ♀ + ♂	80 RA ♀ + ♂
Treatment	8 mL/d for 28 d Echinacin (*E. purpurea* juice)	28 d *E. purpurea*	8 wk Echinaforce (*E. purpurea* tablet)
Study Design	Separate treatment and placebo groups	Pre-to-post comparison	Treatment tolerability study
Exercise	Competitive sprint triathlon at Day 28	3, 30s serial Wingate tests at Days 0 and 28	Subjects' own regular training regimens
Immune System-Associated Outcomes	*Vs. control*: no respiratory infections (placebos had some); ↓ serum and urine IL-2R; ↑ urine IL-6; slight changes in NK cells and T-cells	*Vs. pre-treatment:* reduced symptom severity (but not incidence); reduced post-exercise declines in salivary IgA (SIgA)	*Vs. a reference general population*: fewer upper respiratory infections

Owing to the sparse literature available, many athletics-associated claims about herbal supplements have yet to be scientifically addressed and recommendations need be cautious. As therapeutic

immunomodulators for athletes, there is some evidence that echinacea may be efficacious whereas the evidence for ginseng is murky. Scant literature exists on the potential immunomodulatory effects of the other herbal supplements in Table 2 among athletes or even in the general population, and reviews may be found elsewhere (78).

Controversies and Future Directions

For some herbal supplements, data are only available from animal models or *in vitro* work with human or animal cells. While valuable, such data may not directly translate to human clinical outcomes because of bioavailability/pharmacokinetic reasons or species differences. Potential immunomodulatory effects of bystander molecules (such as endotoxin [LPS] from bacteria growing on plant material or incorporated during the extraction process) are concerns for *in vitro* studies and may explain contradictory activities of plant extracts such as dual cytokine-enhancing and -suppressing properties from a single extract (387); some experiments proactively addressed such concerns whereas others did not. Unaccounted "pre-clinical factors" (especially those during plant growth, harvest, and processing) and differences in experimental methods confound cross-study comparisons (357).

Muddying the waters are issues related to the products themselves: herbal supplement labels may not accurately represent actual supplement contents; there can be lot-to-lot variation from a single manufacturer; and stark differences can exist across manufacturers for a single supplement. Supplements may also inadvertently (or, some allege, covertly) contain substances considered banned/"doping agents" (92,135,214,297). Many herbal supplements are not regulated by government agencies.

Thus, one should be cautious in concluding that any given supplement is consistently efficacious, or that there is a signal failure due to a lack of consiliency in the published research. Rather, lack of consiliency represents the Byzantine nature of herbal supplements in "real world" contexts due to the factors just described. It also limits the guidance professionals can provide to athletes concerning herbal supplement efficacy or safety.

Aforementioned pitfalls can guide future work, which will need to be transdisciplinary to account for all pre-clinical and clinical factors that may influence immune outcomes. Few human *in vivo* studies have focused on specific immune parameters such as cell subpopulations or antibody or cytokine profiles. Such work would help illumine mechanisms and provide the additional benefit of linking clinical outcomes with findings from *ex vivo* or *in vitro* studies.

POLYPHENOLS

Introduction to Polyphenols

The plant kingdom uses nearly 50,000 secondary metabolites for defence, attraction, and protection (163). These plant metabolites include approximately 29,000 terpenes, 12,000 alkaloids, and 8,000 phenolics. The 8,000 phenolic compounds or polyphenols are divided into four main classes: flavonoids (~50% of all polyphenols), phenolic acids, lignans, and stilbenes. Flavonoids are further classified into six simple (flavan-3-ols, flavanones, flavones, isoflavones, flavonols, anthocyanins) and two complex subgroups (condensed tannins or proanthocyanidins, derived tannins) (17) (Table 4). In foods, flavonoids, lignans, and stilbenes are usually found as glycosides, and phenolic acids as esters with various polyols, and structural variations influence absorption and bioavailability (429).

Nutritional assessment of dietary polyphenol and flavonoid intake has improved with the development of databases from Phenol Explorer (www. http://phenol-explorer.eu/) and the U.S. Department of Agriculture (http://www.ars.usda.gov/services/docs.htm?docid=24953). Recommendations for dietary polyphenol and flavonoid intake have not yet been established but should be forthcoming as improvements in assessment methods continue. In Europe, the average dietary polyphenol intake has been estimated at 1,187 mg/day (ranging from about 1,700 mg/day in Denmark to 660 mg/day in Greece), with coffee, tea, fruits, and wine as the principal sources (429). In Europe, only ~100 polyphenols are consumed at levels exceeding 1 mg/day, and flavonoids represent 40% of the total polyphenols ingested (429). Dietary flavonoid intake from all foods and beverages among US adults is 251 mg/day, with tea as the primary source (80% of total flavonoid intake) (355). Only 29% consume tea on a given day, and when tea is removed from the analysis, total flavonoid intake falls to about 50 mg/day, reflecting the low intake of fruits and vegetables by US adults (~2 servings/day) (185,355).

Many flavonoids exhibit strong anti-inflammatory, antioxidant, anti-pathogenic, and immuno-regulatory properties when studied using *in vitro* procedures (1,128,241,413). Most flavonoids are poorly absorbed in the human small intestine and undergo extensive biotransformation after ingestion. Thus, *in vitro* data using the original food-based flavonoid molecule has questionable relevance when evaluating bioactive effects following ingestion. A large proportion of ingested plant polyphenols reaches the colon, and microbial degradation transforms the extremely diverse population of dietary polyphenols into a smaller number of metabolites, including simple phenols and derivatives of benzoic acid, phenylacetic acid, mandelic acid, phenylpropionic acid, and cinnamic acid (116,119,120,345). The bacterial transformation of food polyphenols in the colon varies widely depending on the unique gut microbiota composition of the individual as influenced by genotype, diet, lifestyle, and other factors (116). The metabolites created from bacterial degradation can exert local health benefits to colon endothelial cells, modulate the composition of the microbiota, and hence indirectly influence their own metabolism and bioavailability (116). The gut-derived phenolics can be reabsorbed into the portal vein, undergo phase II biotransformation in the liver, enter the systemic circulation and become a part of the so-called "food metabolome", exert a variety of bioactive effects, and then finally be excreted in the urine (119,120,345) (see Figure 1). Recent *in vitro* studies using biotransformed phenolics at physiologically relevant concentrations indicate that degradation of flavonoids to simpler phenolics actually increases their overall anti-inflammatory bioactivity (241,413).

Epidemiological studies support a strong linkage between high versus low dietary polyphenol intake and reduced risk for overall mortality (189) and a wide spectrum of health conditions including neurodegenerative diseases (371), body weight gain (29), systemic inflammation and oxidative stress (11,72), diabetes (391), cardiovascular disease (409), and hypertension (223). A higher intake of flavonoids predicts increased odds of healthy aging (344). A systematic review and meta-analysis showed that flavonoid supplementation (range of 0.2 to 1.2 g/day in 14 selected studies) decreased URTI incidence by 33% compared with control (372). Many flavonoids exert anti-viral effects, modulate NK cell activities and regulatory T (Treg) cell properties, and influence macrophage inflammatory responses (209). High dietary intake of flavonoids has been linked in the Framingham Heart Study Offspring Cohort with decreased systemic inflammation using a cluster of biomarkers (72).

Countermeasure Effects of Polyphenols to Exercise-Induced Physiological Stress

Taken together, cell culture and epidemiological data support the recent focus of investigators on the use of polyphenols as potential countermeasures to exercise-induced inflammation, oxidative stress, immune changes, illness, and delayed onset of muscle soreness (DOMS) (for reviews, see (268,277,278,379)). Multiple dosing strategies have been employed including single and combined purified polyphenols (e.g., quercetin, resveratrol), plant extracts (e.g., green tea, black currant, pomegranates), and increased fruit and vegetable food or juice intake (e.g., blueberries, bananas, tart cherry juice). Most studies incorporate a one to three week polyphenol loading period prior to an exercise stress intervention. Few papers are available for any particular polyphenol or plant extract, and research designs vary in regards to the supplementation regimen, form of exercise stress, and outcome measures (268,277,278,281,282,379). The data in general support that polyphenol-rich plant extracts and unique polyphenol-nutrient mixtures (e.g., quercetin with green tea extract, vitamin C, and fish oil, or freeze-dried blueberry powder with green tea extract) have small but significant effects in increasing anti-oxidant capacity, with inconsistent, short-term effects on mitigating exercise-induced oxidative stress, inflammation, and immune dysfunction. High blueberry and green tea flavonoid versus placebo intake for 17 days was linked to reduced *ex vivo* viral replication in blood samples collected from athletes after a 3-day overreaching, running protocol (1). Large-dose intake of single flavonoids (e.g., 500 to 1,000 mg quercetin) has been linked to reduced URTI in athletes, but has not proved to be a useful alternative to ibuprofen in regards to countering post-exercise pain, inflammation, and soreness for the athlete (277).

Table 4. Polyphenol classes and subclasses, and food sources.

FLAVONOIDS	**Sample Polyphenols**	**Food Sources**
Simple Flavonoids		
Flavan-3-ols	(+)-catechins, (-)-epicatechin (-)-epigallocatechin-3-gallate	Tea, chocolate, tree fruits, grapes
Flavanones	Hesperetin, Naringenin, Eriodictyol	Citrus fruits and juices
Flavones	Luteolin, Apigenin	Parsley, celery seed, oregano
Isoflavones	Daidzein, Genistein, Glycitein	Soybeans, soy-based foods, legumes
Flavonols	Quercetin, Kaempferol, Myricetin, Isorhamnetin	Onions, apples, tea, berries
Anthocyanidins	Cyanidin, Delphinidin, Malvidin, Pelargonidin, Peonidin, Petunidin	Most berries, stone fruits
Complex Flavonoids		
Condensed Tannins (Proanthocyanidins)	Procyanidins Prodelphinidins Propelargonidin	Chocolate, stone fruit (apples, pears), grapes, strawberries, cranberries nut skins, cinnamon, beer, wine barley, legumes
Derived Tannins	Theaflavins Theabrownins Theaflavins	Fermented teas (black, oolong)
PHENOLIC ACIDS		
Hydroxycinnamic acids	5-Caffeoylquinic acid 4-Caffeoylquinic acid 3-Caffeoylquinic acid Ferulic acid 5-Feruloylquinic acid	Coffee, grain products
Hydroxybenzoic acids	5-O-Galloylquinic acid Gallic acid	Tea, wine, mixed fruits
LIGNANS	Secoisolariciresinol Matairesinol Pinoresinol Lariciresinol Sesamolin	Grain products, nuts, dried fruits, carrots, dried basil, berries, seeds, citrus fruits, stone fruits, peppers, zucchini
STILBENES	Resveratrol	Wine, berries, grapes

Source: Adapted from (17): Balentine DA, Dwyer JT, Erdman JW Jr, Ferruzzi MG, Gaine PC, Harnly JM, Kwik-Uribe CL. Recommendations on reporting requirements for flavonoids in research. Am J Clin Nutr 101:1113-1125, 2015; Phenol Explorer (http://phenol-explorer.eu/compounds).

Future Directions

Future studies should focus on the long-term relationship between increased intake of polyphenols, gut-derived phenolics, and systemic and post-exercise inflammation, oxidative stress, anti-viral defence, and immune function in athletes using global and targeted metabolomics (281,282). Intense and prolonged exertion has been related to an enhanced translocation of gut-derived phenolics into the circulation during a 17-day period of polyphenol supplementation (282) (Figure 1). Elevated blood and tissue gut-derived phenolics from chronic, high polyphenol intake over several months may result in subtle but important bioactive effects that translate to improved recovery and ability to train intensively, with reduced rates of illness (1,241,281,282). This is a complex relationship that demands a multi-omics, long-term approach. Research is needed to define optimal dosing regimens and whether

increased intake of foods high in polyphenols such as berries, tea, and coffee results in meaningful bioactive effects without the need for high doses of unique flavonoid mixtures. In the very least, long-term, high polyphenol intake from plants food sources is important for the health of all humans, including and more importantly, in relation to the present series, for athletes.

PROBIOTICS – PREBIOTICS

Background

Probiotic-rich foods and supplements contain non-pathogenic bacteria that colonise the gut purportedly yielding a variety of health benefits that include reduced incidence of respiratory and gastrointestinal illness. There are several possible ways in which probiotics may reduce the risk of respiratory and gastrointestinal illness symptoms. By their growth and metabolism, probiotics help inhibit the growth of other bacteria, antigens, toxins and carcinogens in the gut, and reduce potentially harmful effects. Probiotics can also influence immune function via interaction with immune cells associated with the gut. Prebiotics are non-digestible food ingredients that promote the growth of beneficial microorganisms in the intestines. Probiotics are found in several foods, particularly dairy products such as milk, yoghurt and cheese (134), although concentrations are relatively low. Consequently, there is widespread interest in use of dietary supplements containing probiotics in both the general and sporting communities.

Figure 1

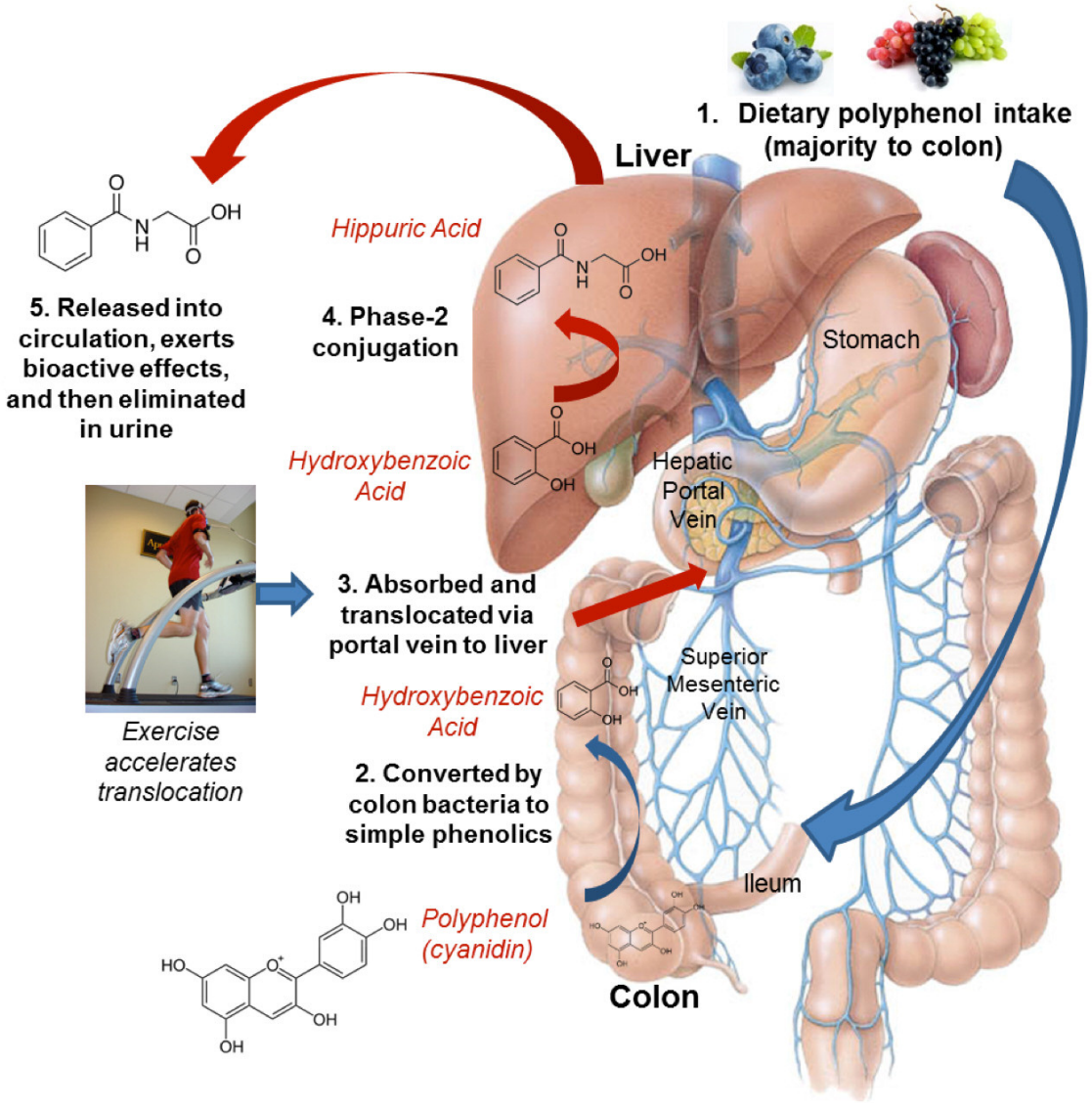

Figure 1 - Cyanidin-3-glucoside (C3G) is a widely consumed dietary anthocyanin, and can be used as a sample polyphenol to show how the body metabolizes flavonoids. After ingestion, the majority of C3G goes to the colon where bacteria degrade it to multiple, simple phenolics including hydroxybenzoic acid. Next, hydroxybenzoic acid and other phenolics are absorbed through the colon to the liver via the superior mesenteric and hepatic portal veins. Prolonged and intensive exercise accelerates the translocation of simple phenolics from the colon to the liver. In the liver, phase II metabolism adds glycine to convert hydroxybenzoic acid to hippuric acid that is released into the circulation. Hippuric acid has been linked to multiple bioactive effects, and is ultimately eliminated from the body through the urine. Many other polyphenols go through a similar biotransformation pathway.
SOURCES: Based on data from references (128) and (281). The contribution of Xiaowei Chen in designing this figure is acknowledged.

Consensus

In clinical practice, probiotics have been used since the early 1900s to manage common gastrointestinal conditions including stomach cramps, irregular bowel movements, excessive flatulence, diarrhoea, and irritable bowel syndrome. In research settings, the focus has been on verifying the clinical benefits of probiotic ingestion and supplementation, and underlying mechanisms of action. Many studies have been conducted on the effects of probiotic use on gastrointestinal problems and URTI in the general population. A recent systematic review (210) of twenty placebo-controlled trials concluded that probiotic use resulted in lower numbers of illness days, shorter illness episodes and fewer days absence from day care/school/work. The most recent Cochrane systematic review of probiotic benefits for URTI using data from randomised controlled trials involving 3,720 non-athletes from 12 studies concluded that probiotics were better than placebo in reducing URTI incidence by ~47%, and the average duration of an acute URTI episode by ~2 days (162).

The most important mechanisms of probiotic action are thought to be via immunomodulation of local immunity (by interaction with gut-associated lymphoid tissue and maintenance of gut barrier function) and systemic immunity (by enhancing some aspects of both innate and acquired immune responses) (31,227). Probiotic intake can increase NK cell cytolytic activity (330), enhance phagocytic activity and microbicidal capacity of granulocytes and monocytes, modify the production of cytokines and elevate levels of specific IgG, IgA and IgM (162), with effects that can extend beyond the gut to distal mucosal sites. Animal studies indicate that regular probiotic ingestion can influence responses in the respiratory tract and improve protection against bacterial and viral pathogens via modulation of lung macrophage and T-cell numbers and functions (131,227,245).

Controversies

To date there are few published studies of the effectiveness of probiotic use in athletes and team sports; a recent comprehensive review (323) identified 15 relevant experimental studies that investigated immunomodulatory and/or clinical outcomes. Of the eight studies that recorded self-reported URTI incidence, five found reduced URTI frequency or fewer days of illness (94,142,167,415,416), and three reported trivial or no effects (141,206,386). A randomised, placebo-controlled trial involving physically active individuals (415) reported that fewer URTI episodes (relative risk ratio 0.73) were experienced in those who ingested daily a *Bifidobacterium* probiotic compared with placebo over a 150-day intervention period. However, a large study of 983 Finnish military recruits failed to show significant clinical benefits for supplementation with a combination of *Lactobacillus rhamnosus* GG (LGG) and *Bifidobacterium animalis ssp. lactis* BB12 for 150 days (203). Studies that examined immunomodulatory effects of probiotics in athletes have reported increased interferon-γ production in whole blood culture (83) and T-cells (94) and better maintenance of secretory IgA during intensive training (142,242). A recent study reported that URTI incidence was unchanged despite reductions in cytomegalovirus (CMV) and Epstein-Barr Virus (EBV) antibody titres after 20 weeks of supplementation with *Lactobacillus casei* (144).

Most studies have examined probiotic effects in small numbers (<50) of recreationally active individuals over periods lasting <6 months. URTI has typically been established by self-report questionnaires and not all studies have used a randomised, placebo controlled design. However, there is now sufficient understanding of the mechanism of action of certain probiotic strains, and enough evidence from trials with athletes and highly physically active people (in addition to 12 studies cited in The Cochrane review (162) on children and adults) to signify that there are mostly positive effects. Similar to other supplements for which health claims are made there is concern of bias in the literature, with a stronger likelihood of publication of studies with positive, as opposed to trivial, equivocal or negative outcomes.

Other potential benefits of probiotics could be reduced risk of gastrointestinal discomfort symptoms and diarrhoea (e.g. so-called runner's trots) during prolonged exercise, reduced endotoxaemia during exercise in the heat, and reduced incidence of gastrointestinal infections – a particular concern when travelling abroad. Further large-scale studies are needed to determine if these potential benefits are real, and to confirm that taking probiotics can reduce the number of training days lost to infection and which strains of probiotics are most effective for athletes. The studies that have shown reduced URTI incidence in athletes have been mostly limited to *Lactobacillus* and *Bifidobacterium* species and used daily doses of ~10^{10} live bacteria. These doses (~10^{10} live bacteria) showing efficacy with athletes are comparable to those used in non-athlete studies (range ~10^{8}-10^{10}) although recommended dosages can be strain-specific. Although probiotic supplements contain similar bacterial species to dairy foods, there is little consensus on the relative effectiveness of commonly used species. As for prebiotics, or combinations of prebiotics and probiotics, there are currently no published studies on their efficacy in athletes for reducing respiratory or gastrointestinal illness symptoms.

Future Directions

Long-term tolerance of probiotic supplementation in highly-trained athletes over several months to years or the benefits, if any, of cycling on and off probiotics, are important questions warranting investigation. The laboratory-based efficacy and field effectiveness of multi-component formulations combining several different probiotics species, or probotics and prebiotics, need evidence-based studies. Pharmaceutical companies are already making a wide range of multi-component formulations. No studies have systematically investigated how the dosage regimens of probiotic supplementation might vary as a function of sex, age, medical history, dietary practices, fitness level and/or training background. Health care practitioners are seeking this information to assist them in prescribing individualised probiotic/prebiotic supplementation programmes.

BOVINE COLOSTRUM

Background

Bovine colostrum is the fluid produced by the mammary glands for 24-72 hours following calving. While antibody

transfer in a human takes place predominantly via the placenta, calves rely on colostrum for the passive transfer of immunoglobulins (Ig). As such, the concentration of Ig and other immune factors, in combination with growth factors and nutrients, is much greater in bovine than human colostrum. For the calf, bovine colostrum is essential for immune system establishment and gastrointestinal growth and differentiation. This has led researchers to explore the potential of bovine colostrum to modulate immune function in humans, particularly in exercise where immune perturbations are common.

Bovine colostrum is an incredibly complex fluid and understanding of its potential molecular function is improving with advances in technology, such as proteomic analysis (8). Bioactive components of bovine colostrum include IgG, lactoferrin, lactoperoxidase, defensins, trypsin inhibitor, micro RNAs, insulin-like growth factor-1 (IGF-1) and transforming growth factor-β (190,343). Following ingestion, many of these components have been shown to survive digestion and exert effects at the level of the gut, or systemically. In cell culture and animal models (porcine, rat and mice) bovine colostrum exhibits anti-bacterial, anti-inflammatory and antiviral properties (425,427). However, the effectiveness of stable, standardised preparations of non-hyperimmunised bovine colostrum to modulate the immune system in healthy, exercising humans is less clear.

Consensus

Exercise is associated with immune perturbations and upper respiratory symptoms (URS) are commonly reported in elite athletes (122). Several investigations have reported URS incidence following a period of bovine colostrum supplementation, with all of them suggesting that bovine colostrum supplementation is associated with a reduction (not always statistically significant) in URS incidence in athletes. A retrospective analysis of training diaries from investigations with healthy, active male participants (n=174) reported a significantly lower incidence of URS with eight weeks of bovine colostrum supplementation of 60 g/day (32%) compared to a whey placebo (48%) [relative risk (RR) of 0.6](49). Similarly, when compared to a whey or skim milk powder placebo, others have reported a trend for a reduction in URS incidence over 8-12 weeks of lower dose colostrum supplementation (10 to 25 g) in trained cyclists [RR 0.4 (362) and 0.3 (363)], elite swimmers (weeks 5-10: RR 0.4) (95), active males (RR 0.6) (198) and marathon runners (mean URS incidence of 0.8 in colostrum group compared to 1.1 in placebo) (96). The ability of bovine colostrum to shorten URS duration is less clear, with some investigations reporting no change (49,198,362) and some a reduction (95,96,363). While the ability of bovine colostrum to shorten symptom duration is unclear, bovine colostrum supplementation (10-60 g) for greater than four weeks appears to reduce self-reported URS incidence by 30 to 60%.

While changes in salivary SIgA have been related to URS (137) the mechanism for a reduction in URS following a period of bovine colostrum supplementation does not appear to relate specifically to increases in SIgA concentration (101,199,363). One investigation reported a significant 79% increase in SIgA concentration after 12 weeks of bovine colostrum supplementation (26 g/day) (d=4.8) (96), although the placebo group also reported a large increase over this timeframe (d=3.0). While some of the increase in SIgA was attributed to competing in a marathon, colostrum accounted for 29% of the variation in SIgA (96). In contrast, studies of similar duration and/or those providing higher doses of bovine colostrum (~60 g/day) have not reported changes in SIgA (198,256,362,363). Other proposed mechanisms for the reduction in URS incidence may be related to minimising the increase in winter salivary bacterial load (198) or a reduction in the suppression of receptor-mediated stimulation of neutrophil oxidative burst (199). There is also evidence that bovine colostrum may reduce the post-exercise decrease in neutrophil function and salivary lysozyme (101), and reduce immunodepression during a period of intensified training (362) although these mechanisms are yet to be confirmed. It is unlikely that bovine colostrum supplementation alters circulating cytokine concentrations following short-term intense exercise (68,362) in athletes who have not undertaken a period of intensified training overload.

Controversies

The gastrointestinal tract is the largest immune organ in the human body and in combination with its intestinal microbiota it not only provides a physical barrier against commensal and pathogenic bacteria, but plays a central role in innate and adaptive immunity (364). In the calf, bovine colostrum is essential for intestinal development, providing a rationale to investigate the potential of bovine colostrum to influence gut health in humans.

Early work in healthy adults demonstrated that bovine colostrum reduced gastrointestinal permeability when co-ingested with non-steroidal anti-inflammatory drugs (314) and reduced systemic endotoxin concentrations in abdominal surgery patients (44), suggestive of maintenance of the intestinal barrier preventing endotoxin translocation across the gut. Only two human studies have investigated if bovine colostrum reduces the increase in intestinal permeability associated with exercise (240,264) and their findings are conflicting, possibly attributable to study differences in markers of intestinal permeability, exercise protocols, colostrum dose and period of supplementation. While animal studies suggest a beneficial effect (321), more controlled human studies are required to determine the impact of bovine colostrum on exercise associated gut permeability and endotoxin translocation.

In combination with influencing gastrointestinal growth, IGF-1 plays a role in immune and neuroendocrine regulation. While only one study has reported autonomic alterations following a period of colostrum supplementation (363), controversy exists as to the possibility of absorption of IGF-1 from bovine colostrum. Only one laboratory has reported increases in IGF-1 following bovine colostrum supplementation periods of eight days and of two weeks (255,256) with others reporting no change (53,90,228), and athletes ingesting 60 g/day not returning any positive doping test (for substances banned in 2002) (217). Mero determined that orally administered IGF-1 alone did not appear in the circulation and concluded that IGF-1 is not absorbed from bovine colostrum (255) although this is not necessarily correct as bovine colostrum contains

numerous components that would keep IGF-1 intact during gastrointestinal transit (315). The IGF-1 content in a 20 g dose of bovine colostrum (343) is approximately equivalent to that contained in three glasses of milk. Although not on the World Anti-Doping Agency's list of banned substances, this governing body does not recommend the ingestion of colostrum.

Future Directions

While bovine colostrum appears to reduce the incidence of URS, the specific mechanism/s for this require further investigation and should include measures of salivary antimicrobial proteins, in addition to SIgA. Well-designed investigations are also necessary to elucidate the potential of bovine colostrum to modulate intestinal permeability and inflammation in exercising humans.

As more is discovered about the minor constituents of bovine colostrum (8), there is the potential to discover novel bioactive proteins, and enhance understanding of the efficacy of already known components. Lactoferrin, isolated from bovine colsotrum, shows particular promise as an immune modulating glycoprotein. The majority of orally administered bovine lactoferrin survives gastric transit (392), exerting its effects through the interaction with gut enterocytes and resident immune cells. In neonatal animal models lactoferrin stimulates crypt cell proliferation (333), increases serum IgG and modulates cytokine secretion of stimulated mesenteric lymph nodes and spleen immune cells (89). Animal and cell culture models support the anti-viral, anti-bacterial immune modulating properties of lactoferrin (399) so it is suprising that there is limited literature investigating the effects of bovine lactoferrin supplementation in healthy humans (266), and no studies investigating potential effects on exercise-induced immune modulation. Benefits of lactoferrin for Fe deficiency anaemia associated with pregnancy (301), and a reported increase in T cell activation in healthy males (unblinded study) (266) support the exploration of bovine lactoferrin supplementation to modulate exercise-induced immune perturbations and/or enhance immune surveillance.

VITAMIN D

Introduction

From a sport and exercise science perspective, interest and research into vitamin D over the last decade has witnessed a remarkable resurgence (420). The reason for this is partly attributable to the re-emergence of the entirely preventable bone disorder rickets (298) but perhaps mainly due to the emerging evidence to suggest a fundamental role of vitamin D in many areas pertinent to the athlete. These include: skeletal muscle function (84), body composition (172), inflammation (421), muscle regeneration (299), and cardiac structure as well as aspects of innate and acquired immunity (168).

Vitamin D is unique in that, unlike other vitamins, it is not primarily obtained from dietary sources; rather it is synthesized via UV irradiation of the skin's dermis. Once in the circulation, either from diet, supplements or UV irradiation, vitamin D is transported to the liver bound to vitamin D binding protein where it is hydroxylated eventually leading to its activation. The hydroxylated compound 25-(OH) D is the major circulating vitamin D metabolite and is therefore the assay of choice when it comes to detecting vitamin D deficiencies. A further hydroxylation step in the kidney (or some tissues directly) is required to produce the active metabolite 1,25-$(OH)_2$ D and it is this active metabolite that is responsible for the multiple biological effects via both genomic and non-genomic mechanisms.

Defining terminology and why deficiencies occur

Although the multiplicity of fundamental biological roles of vitamin D is now appreciated, it is well documented that many individuals, including elite athletes, exhibit vitamin D deficiencies (Figure 2). These deficiencies are clear in athletes who live in temperate (84) as well as sunny climates and train predominantly in both indoor (424) and outdoor environments (84). This worldwide phenomenon of vitamin D deficiencies is partially attributable to poor dietary intakes, although the major reason is more likely a direct consequence of modern sun-shy lifestyles, including the use of appropriately applied high-factor sunscreen creams (125), which significantly restrict vitamin D synthesis.

A major area of confusion in the vitamin D literature arises from a lack of consensus as to what constitutes a genuine vitamin D deficiency and, more recently, the concept of a potentially "optimal" vitamin D concentration for health and athletic performance. It is beyond the scope of this article to explore this debate and therefore an "adequate" concentration will be classed here as >50 nmol/l as defined by the US Institute of Medicine. Moreover, it has also been suggested that what may be optimal for one tissue, such as bone or skeletal muscle, may not be optimal for another, such as immune function. In fact, from an exercise immunology perspective recent research is beginning to indicate that aspects of the immune system may require higher concentrations of vitamin D than has previously been defined as "adequate" for bone health (168).

Vitamin D and the immune system

Emerging research suggests that vitamin D plays a key role in both innate and acquired immunity, most likely exerting its function through gene expression modulation (168,183). In this role 1,25-$(OH)_2$ D functions as part of a heterodimer with its vitamin D receptor (VDR) and the retinoid X receptor modulating the expression of genes with specific vitamin D response elements located in the regulatory region (168). In fact it is estimated that close to 5% of the human genome is modulated by vitamin D (430), which is required in sufficient quantities to work effectively in gene expression modulation.

Many cells of the immune system including monocytes, macrophage, neutrophils and T and B lymphocytes contain the VDR and also express the enzyme, 1-α hydroxylase, which is responsible for hydroxylation of 25-(OH) D to its active 1,25-$(OH)_2$ D form. Activation in immune cells appears to be regulated by circulating concentrations of 25-(OH) D and induced by activation of the toll-like receptor cascade in the presence of pathogenic microbiota (30). In the immune system specifically, vitamin D up-regulates gene expression of broad-spectrum anti-microbial peptides (AMP), important regulators in innate immunity (231,408), and exerts an

Figure 2

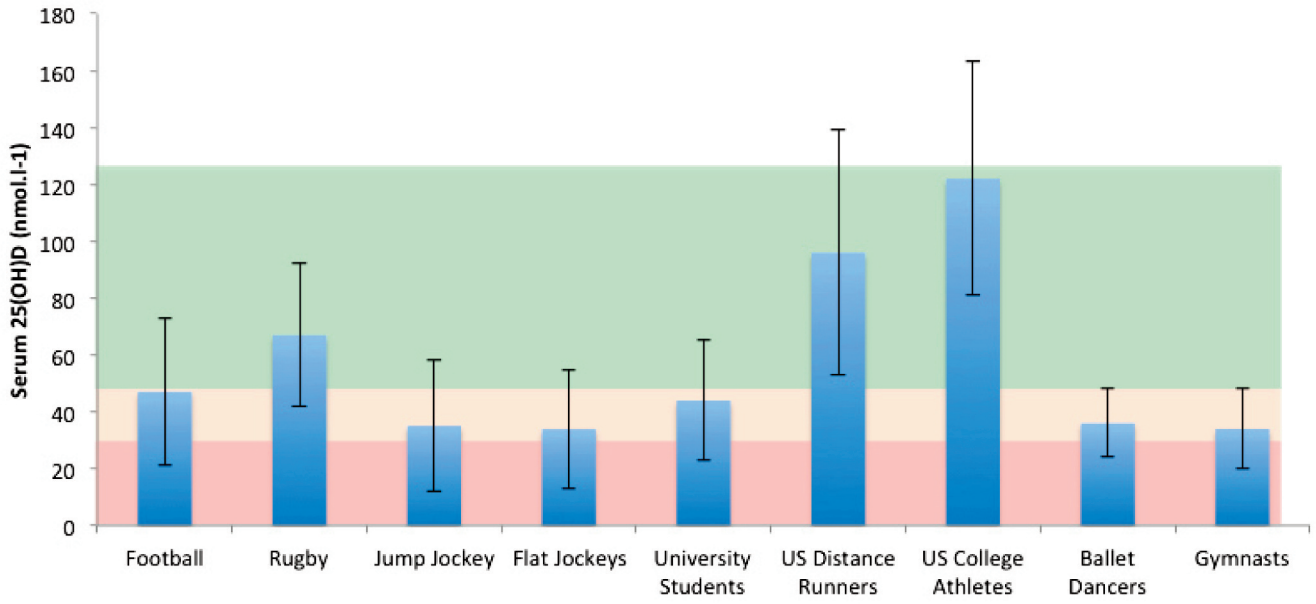

Figure 2 – Vitamin D concentrations of a variety of athletes tested (at rest) internationally. Pink area represents deficiency (<30 nmol/l), yellow area insufficiency (<50 nmol/l) and green area adequate (>50 nmol/l) as defined by the US National Institutes of Health Office of Dietary Supplements. (Data redrawn from (84,157,226,421,424)).

immunomodulatory effect on T and B lymphocytes in acquired immunity (168,431). AMP, including cathelicidin, are important proteins in the innate immune system (148) and help defend against acute illness including tuberculosis, influenza and the common cold (64,65,395). It is further suggested that vitamin D maintains a balance between the inflammatory Th1/Th17 cells and the immunosuppressive Th2/Treg cells to dampen inflammation and tissue damage (178) and to modulate the acquired immune response. Additional studies suggest that vitamin D enhances natural killer cell cytolytic activity (4), and acts to trigger the oxidative burst in activated macrophages (368). A single dose of vitamin D3 (100,000 IU) has been shown to enhance the innate immune response and restrict growth of mycobacteria *in vitro* (248).

Variations in vitamin D concentrations have the potential to influence immune response. A handful of studies in athletes (93,168), military personnel (218) and the general population (28,136,342) have reported negative associations between vitamin D concentration and incidences of URTI. In one study in college athletes, vitamin D concentrations over the winter and spring were negatively associated with documented frequency of acute URTI (157). The breakpoint for contracting single illness appeared to occur at ~95 nmol/l such that all athletes with circulating concentrations lower than this breakpoint had one or more episodes of illness whereas those with higher concentrations had one or fewer episodes. As has been shown in Figure 2, many athletes present with vitamin D concentrations significantly below this proposed breakpoint. A similar study in endurance athletes reported that a greater proportion of athletes maintaining circulating 25-(OH) D concentration <30 nmol/l presented with URS with the fewest symptoms reported in those with 25-(OH) D concentrations >120 nmol/l (171). Athletes with low vitamin D concentrations also had more URS days and higher symptom-severity scores. Randomly-assigned, placebo-controlled studies are needed in athletic populations to confirm the effectiveness of correcting low vitamin D concentrations on aspects of immune health and the prevention of URTI. A recent study in university athletes found evidence that 14-week supplementation with 5000 IU per day of vitamin D_3 during winter training significance increased salivary secretion rates of cathelicidin and SIgA (171).

Consensus

It is now established that many athletes present with deficient D concentrations, especially during the winter months, which result in numerous deleterious consequences. The concept of an "optimal" vitamin D concentration, however, is proving difficult to establish with emerging evidence suggesting that "optimal" vitamin D concentrations may in fact be tissue specific. Recent evidence is suggesting that athletes presenting with "sufficient" vitamin D concentrations (>50 nmol/l) may still be at an increased risk of contracting a URTI compared with athletes presenting with >75 nmol/l (171).

It must be stressed that there is a growing trend for mega dose supplementation in athletes. This is of concern due to some evidence which suggests an increase in all-cause mortality in individuals with high (>140 nmol/l) vitamin D concentrations (118), although cause and effect data to prove this are still lacking.

At present, perhaps the best advice for athletes is to monitor their vitamin D concentration and, in terms of immune health, aim for a target concentration >75 nmol/l. During the summer months athletes should aim to obtain sensible sun exposure, whilst ensuring that they do not suffer from sunburn, and consider a supplement of up to 4000 IU vitamin D_3 per day during the winter months if concentrations drop below 75 nmol/l.

Future directions

Future studies now need to establish if there is in fact an optimum concentration for an athlete's immune health utilizing a research design that will allow cause and effect to be established rather than reliance on correlative data.

IMMUNONUTRITION AND EXERCISE IN SPECIFIC POPULATIONS

Competitive athletes and military personnel

Background

A commonly asked question is, 'do athletes and military personnel have special nutritional requirements to maintain immunity; for example, do they need 'immune-boosting' supplements?' A somewhat simplistic answer is 'no'; so long as the diet meets the energy demands and provides sufficient macro- and micro- nutrients to support the immune system (140,405). The reasoning here is mostly based on the empirical evidence along with some sound logic: support for 'immune-boosting' nutritional supplements largely comes from studies in those with compromised immunity, such as the elderly and clinical patients; not, from young, otherwise healthy, individuals whom, as logic would dictate, might have little to gain from such supplements (229). To accept this viewpoint ignores the proviso about matching energy intake to expenditure and providing sufficient macro- and micronutrients to maintain immunity. Indeed, there are various athlete and military scenarios where energy, macro- and micronutrient intake may be insufficient.

Consensus

In the real world, athletes and warfighters either intentionally or non-intentionally experience deficits in energy intake (e.g. weight-loss diets, restricted rations) and macronutrient intake (e.g. restricted carbohydrate). There is substantial evidence showing that only a few days on restricted energy intake compromises immunity (221,295,401). There may be other times when athletes or warfighters experience both a down-turn in host defence and increased exposure to pathogens, e.g. foreign travel for training camps, competitions and military operations. As such, there are specific scenarios when these individuals might benefit from nutritional supplements to bolster immunity (Table 5). Thus the current, more reasoned, answer to the question, 'do athletes and military personnel have special nutritional requirements to maintain immunity?' is 'yes, sometimes.'

Table 5. Effects of nutritional supplements on the common cold and immunity in athlete and military scenarios

Scenario	Immune health and performance	Supplement	Supporting evidence and knowledge gaps	Refs.
Winter season	Common cold and Influenza season; URS decrease training and performance; low UVB skin exposure decreases vitamin D	Vitamin D_3	Moderate support for vitamin D in athletes/military; recommend 1,000 IU/day D_3 from autumn to spring to maintain sufficiency	168
		Vitamin C	Moderate support in athletes/military; Cochrane review of 5 studies in heavy exercisers (n=598) shows ~50% decrease in URS taking vitamin C (0.25–1.0 g/day); unclear if antioxidants blunt adaptation in well-trained; further support required	173
		Probiotics	Moderate support in athletes with daily dose of ~10^{10} live bacteria; Cochrane review of 12 studies (n=3720) shows ~50% decrease in URS incidence and ~2 d shortening of URS; minor side effects	142, 162
		Glutamine	Limited support; Gln (2 x 5 g), or a Gln precursor, decreased self-reported URTI after endurance events; this dose increases but does not maintain blood Gln nor alter many aspects of immunity; mechanism for therapeutic effect unclear; further studies required	77, 139
Suffering URTI	URS decrease training and performance; particularly in illness prone	Zinc lozenges	Moderate support; Cochrane review shows benefit of zinc acetate lozenges (75 mg) to decrease duration of URS; must be taken < 24 h after onset of URS; side effects include bad taste and nausea	367
		Vitamin C	No support; Cochrane reviews show no benefit of 'initiating' vitamin C supplementation (> 200 mg/day) after onset of URS	114, 173
Foreign travel	Increased URS risk; stress prior to travel may decrease immunity; increased exposure to pathogens; travellers' diarrhoea and risk of dehydration	Probiotics	Moderate support; probiotics can reduce risk of travellers' diarrhoea; probiotics do not decrease episode duration; minor side effects; further studies required	232, 380
Energy deficit	Training with energy deficit decreases performance and immunity	Multi-vitamin/ mineral; probiotics; bovine colostrum etc.	Limited support for supplements to reduce URS and bolster immunity in these scenarios; multivitamin/mineral supplement may provide insurance; unclear if antioxidants blunt adaptation in well-trained; impact of train low CHO on immune health remains unclear; further studies required	45, 110, 132
Train low CHO	CHO restriction/periodization may increase adaptation and performance but decrease immunity			86, 165
Training camp/military operation	Threats to immunity include: increase in physical exertion; other stressors e.g. psychological, altered sleep, heat and/or altitude; limited food choices; energy deficit			140, 406, 407

URS = upper respiratory symptoms; URTI = upper respiratory tract illness; CHO = carbohydrate.

Controversies and future directions

Paradoxically, nutritional strategies currently adopted by endurance athletes, including training with low carbohydrate (Table 5), may benefit training adaptations and performance at the expense of immunity; for example, carbohydrate restriction may increase the immunosuppressive stress hormone response to exercise (86,140,165). Consequently, the rather modest benefits studies show in terms of training adaptations

and performance might, in the long term, be lost if the athlete gets sick more often; *viz*. 'the less sick the more the athlete trains and the better they perform' (246,380,407). Studies are required to investigate whether the nutritional practices adopted by elite athletes impair immunity and increase infection; and, whether purported 'immune-boosting' supplements benefit immune health without blunting the desired training adaptations. Recent Cochrane reviews have noted the low quality of many studies on nutritional supplements to support immune health; specifically, small samples, poor controls and unclear procedures for randomisation and blinding were commonplace (162). Clearly, there is a pressing need for randomized controlled trials in elite athletes and military personnel with sufficient participant numbers; rigorous controls and procedures; appropriate supplementation regimens; and, clinically meaningful *in vivo* measures of immunity (see section on biomarkers)

Overweight and obese exercising humans

Obesity, a state of malnutrition related to excessive intake of energy has been related to immune dysfunction (175). High body fat levels are accompanied by changes in white blood cells, especially an increase in total leucocyte number, with altered differential counts in neutrophils, monocytes and lymphocytes, finding increased values of lymphocytes and neutrophils in boys and girls, respectively. However, low T- and B-cell mitogen-induced proliferation is shown in obesity (247). Both cell-mediated and humoral immunity are affected by obesity, with low antibody production after vaccination (247,361). Moreover, obesity has been characterized as a state of chronic low-grade inflammation, with an excessive amount of adipose tissue as the main determinant of this process (411).

Physical inactivity seems to be a prominent and modifiable risk factor to develop excess weight and obesity. According to epidemiological studies and clinical trials there is evidence addressing the influence of physical activity and fitness on low-grade inflammation in adulthood (160,302), athletes (292,390) and, to a lesser extent, in children and adolescents (412). Regular exercise seems to have proven anti-inflammatory effects in normal subjects (412) but also in overweight and obese individuals (175,411).

Regular training leads to a reduced TLR4 expression baseline, accompanied by a lower percentage of circulating CD14+CD16+ monocytes, which could result in an anti-inflammatory effect (243,385). In the case of obese subjects, macrophages are a potential source of inflammatory processes where the microbiota is also involved leading to lower insulin sensitivity in several tissues (liver, adipose tissue, hypothalamus, muscle) and a state of chronic low-grade inflammation (393). Studies performed in mice fed a high fat diet showed that exercise training reduces visceral adipose tissue followed by a change of M1 macrophage phenotype to M2 macrophages (205).

Cytokines have been shown to play a particular role in the regulation of the metabolism due to exercise, leading to immunomodulation within the adaptation mechanisms involved (272). However, it is important to highlight that the cytokine response depends on the acute or chronic exercise as well as its intensity, duration, the mass of muscle recruited, endurance capacity and idiosyncracy of the person practising exercise (411). The contracting skeletal muscle is a major source of circulating IL-6 in response to acute exercise. During heavy exercise, such as a marathon, there can be up to a 120-fold increase in the IL-6 plasma levels with the duration of the event explaining more than 50% of the variation. The aforementioned plasma IL-6 increase supports the hypothesis that post-exercise cytokine production is related to skeletal muscle and duration of exercise. Nevertheless, IL-6 shows a markedly lower response to acute exercise in trained subjects. The health benefits of long-term regular exercise are ascribed to the anti-inflammatory response elicited by an acute bout of exercise, which is partly mediated by muscle-derived IL-6. This IL-6 increase seems to induce an anti-inflammatory cytokine cascade (IL-1ra and IL-10), and to inhibit the production of pro-inflammatory cytokines, such as TNF-α (305,308). Therefore, the anti-inflammatory effects of exercise may offer protection against TNF-induced insulin resistance.

IL-6 stimulates lipolysis as well as fat oxidation. The increase of IL-6 after acute exercise is linked to increased CRP levels (290). In response to regular physical activity, basal as well as post-exercise plasma levels of IL-6 decrease by mechanisms that might include increased glycogen content, improved antioxidative capacity and improved insulin sensitivity. The lower levels of IL-6 in circulation will subsequently result in lower CRP levels. In untrained subjects, basal plasma IL-6 and CRP levels are elevated via mechanisms that may involve impaired insulin sensitivity and/or increased oxidative stress (305,308). The status of glycogen stores is also an important contributor to IL-6 production with exercise: the lower the glycogen the higher the IL-6 production. It is of particular importance in overweight, obese and diabetic patients especially for those with particular diets.

Adipose tissue is regarded as an active endocrine organ that releases a large number of bioactive mediators (pro-inflammatory cytokines, leptin, adiponectin, peptide YY, among others) modulating not only appetite and metabolism, but also the immune system involving inflammatory processes (339).

The EVASYON study aimed to develop a comprehensive intervention including diet and physical activity and to evaluate its efficacy in adolescents with excess weight and obesity. Some beneficial changes were achieved due to an early reduction of immunological and metabolic markers including leptin, IL-8 and TNF-α, delivered by adipose tissue and whose high levels are considered to be linked to an inflammatory state (339).

In the AFINOS study performed in Spanish adolescents, cardiorespiratory fitness and muscular fitness were shown to be inversely associated with adiponectin and leptin levels. Vigorous physical activity levels have also been inversely associated with leptin (249).

Preliminary evidence from the AFINOS study seemed to indicate that achievement of a healthy weight in this population

group might be the most effective strategy to prevent chronic low-grade inflammation and future cardiovascular and metabolic diseases. Indeed, an active lifestyle and a desirable cardiorespiratory fitness may attenuate these problems.

Therefore, physical exercise has been shown to increase weight management efficacy, being a potential therapeutic approach to modulate low-grade inflammation. Particularly, the encouragement of doing physical activity during adolescence could have important implications for public health, as a specific strategy to avoid high levels of the well-known sedentary habits during this crucial life period. Likewise, other types of physical activity related to muscular fitness (that is, resistance training) might be taken into consideration during adolescence because high levels of muscular fitness have shown negative associations with inflammatory proteins. Therefore, understanding the interrelationships between physical activity, fitness and fatness may be the main way to prevent low-grade inflammation, particularly at these ages (250).

The exercising elderly

It is well documented that aging is associated with a decline in cell-mediated immune function, a phenomenon often called immunosenescence, which contributes to the higher morbidity and mortality from infectious diseases in older population. On the other hand, mounting evidence suggests that aging is associated with an increased inflammatory response (212,347,398). Chronic, low-grade inflammation has been implicated in the pathogenesis of many common degenerative and metabolic diseases associated with aging. Several studies have shown that acute and prolonged or vigorous bouts of exercise cause immunodepression as well as the related increase in incidence of URTI symptoms, and may also induce increased inflammation and oxidative stress (273,365,406). Therefore, extreme exercise may exacerbate the age-associated dysregulation of the aging immune system (286,366,394). However, regular moderate exercise in general causes no such adverse effect, and might even enhance the immune function (274), particularly in older individuals (365,406). Studies showed that calisthenic exercise increased NK activity and T cell function in elderly women (286); primary antibody and delayed-type hypersensitivity (DTH) responses to the novel antigen keyhole limpet haemocyanin (KLH) were lower in older than in young subjects, but these *in vivo* measures of the immune function were improved by exercise in older but not young subjects (150,370). A possible reason behind this observation is that, relative to their young counterparts, the older individuals have a less optimal immune response which is restored by moderate exercise (406).

Proper nutrition, *i.e.*, adequate and well balanced intake of nutrients, is important for normal function of the immune system. Currently no information is available as to whether exercising older persons have unique nutritional needs compared to their young adult counterparts. However, a significant percentage of older adults have low consumption of several micronutrients including the B vitamins, vitamin E and Zn, all of which are needed for the normal function of the immune system (239,300). At the same time, both inflammation and oxidative stress increases with aging suggesting that the older exercising adults might require higher level of nutrients and foods with antioxidant and anti-inflammatory properties. In addition, when conducting the same type of exercise, the older persons are known to more easily suffer from muscle damage and require a longer period to recover from it (127). The exercise-induced muscle damage can initiate an inflammatory response, which could further exacerbate the chronic low-grade inflammation observed in older adults, further suggesting that exercising older adults might require higher level of nutrients and other dietary components with immune enhancing and/or anti-inflammatory properties than non-exercising older adults or their young counterparts. However, previous studies which mainly involve younger adults have indicated that consuming antioxidant supplements, with the possible exception of quercetin, does not help in terms of improving exercise-induced immunodepression, inflammatory response, and URTI (see the antioxidants and polyphenols sections – both in this series). In support of this, studies thus far suggest that antioxidant micronutrient supplementation may not afford protection against muscle damage but, rather, it may interfere with cellular signalling functions of ROS and interrupt training-induced adaptations (50,192). Further studies are needed to determine whether exercising older adults would respond in a different manner from young adults, given the observation that older adults may have higher requirements for nutrients and food components that possess antioxidant and anti-inflammatory properties.

Another nutritional consideration for older adults is the amount of total energy. Since the intensity and duration of exercise are usually less in older persons compared to young adults, the total calorie intake should be adjusted accordingly to avoid conversion of excess calories to body fat. Additionally, the general recommendation to increase calorie intake from carbohydrates should be exercised with caution for older persons, due to the fact that glucose tolerance and insulin sensitivity are decreased with aging.

In summary, information on nutritional needs of exercising older adults whether micro or macro-nutrients is scarce. The age-associated dysregulation of the immune response (suppression of cell-mediated immunity and increased inflammation), together with other age associated changes, and low consumption of nutrient rich foods strongly supports the necessity of further research in this area so that specific recommendations can be made.

BIOMARKERS IN IMMUNONUTRITION

Introduction

In this section the strengths and weaknesses of various biomarkers used in studies by nutritional immunologists are evaluated (Table 6). An important consideration is that exercise immunologists often perform investigative work in the field, away from the rigorously controlled laboratory environment. Consequently, the studies are often limited by a lack of experimental control and the choice of measurement tool(s) is often dictated by convenience, practicality and cost. With this in mind, areas of uncertainty, gaps in knowledge and opportunities for continued research development on immune biomark-

ers are highlighted, particularly research targeted towards the development of technologies applicable in the field. These opportunities include rapid, non-invasive measurements of immunity by portable devices at single time points and even continuous monitoring by wearable technology (e.g. smart contact lenses) may be possible in the not too distant future.

Classification of upper respiratory tract illness

Arguably the most illuminating studies on factors influencing common cold incidence (e.g. psychological stress, sleep) have quarantined individuals before and for up to 7 days after intranasal inoculation with live common cold viruses (rhinovirus, respiratory syncytial virus or coronavirus) and assessed the development of clinical colds (Table 6) (87,88). Although this represents a strong experimental model to identify the effects of nutritional interventions on common cold incidence, there are obvious limitations that have prohibited its adoption by exercise immunologists. These include ethical considerations, as well as cost, requirement for medical facilities and support, together with the fact that athletes are unlikely to participate in a study where ~40% of individuals develop a common cold (191). Another limitation is that studies using the common cold challenge model have not identified whether the increased development of common cold in those under psychological stress (88) or sleep stress (87) is due to a systemic immunodepression or local effects at the nasal mucosa. For these reasons exercise immunologists have relied heavily on subjective self-report of common cold symptoms using either unstandardised health logs, standardised symptom questionnaires (e.g. Jackson) or physician assessment of common cold symptoms (Table 6). In 1958 Jackson et al. reported clinical features of the common cold after infecting >1,000 individuals by nasal instillation of nasal common cold secretions collected from donors (191). In the ~40% of individuals who developed symptoms of a common cold in the 6-day monitoring period, 8 clinical symptoms were incorporated into the questionnaire. Symptoms included headache, sneezing, chilliness and sore throat that appeared in the first 48 h and nasal discharge, nasal obstruction, cough and malaise that appeared later. The 8 clinical symptoms were scored on a 4-point scale from 0 (no symptom) to 3 (severe symptom): Jackson's criteria for a common cold included a total symptom score of ≥14 and a "yes" answer to the dichotomous question, 'do you think that you are suffering from a common cold?' during the 6-day monitoring period (191).

The Wisconsin upper respiratory symptom survey (WURSS; (20)) has also been used widely by exercise immunologists (149,283,374), including nutritional intervention studies (149), as it considers the impact of common cold symptoms on quality of life measures (Table 6). Studies have raised questions about the validity of the physician-verified common cold, highlighting that neither self-reported nor physician-verified common colds should ubiquitously be referred to as infectious (93,374). Notwithstanding these limitations, studies highlight the negative impact of self-reported common cold symptoms on training volume (246) and medal-winning prospects in elite athletes (322,380).

Three key recommendations include: 1) standardising the recording of common cold symptoms in athletes (e.g. incorporating the Jackson common cold scale or the WURSS into a training log); 2) recording the impact of common cold symptoms on training and performance (e.g. discontinued or reduced training); and 3) where possible, incorporating identification of infections from pathological analysis of swabs (Table 6 see next page). Common cold challenge studies show that only ~40% of those inoculated develop symptoms associated with the common cold yet >80% are typically infected (positive virology/specific antibody response) (87,88,191). Thus, an important research question for exercise immunologists is, 'why do less than half of those infected develop symptoms of the common cold? Adopting these recommendations will allow the exercise immunologist to understand more fully the influence of exercise training and nutritional interventions on the development of common cold symptoms and their impact on training and performance in those with and without confirmed infectious aetiology.

***In vivo* immunity**

Where feasible, exercise immunologists are encouraged to use *in vivo* methods for assessing immune responses (3,405,406). By initiating an integrated and highly coordinated immune response in the normal tissue environment, *in vivo* immune methods provide more clinically relevant information that extends beyond *in vitro* assays (Table 6) (87). A weakness of many *in vitro* assays is the requirement to separate immune cells from their normal environment and incubate in artificial culture. Examples of *in vivo* immune methods include assessing: the circulating antibody response to influenza vaccination and hepatitis B vaccination (55); the local skin response to intradermal antigens using delayed type hypersensitivity (DTH) (52) and to topically applied antigens using contact hypersensitivity (CHS) (164). Studies demonstrate that both acute and chronic exercise can increase influenza vaccination success (circulating antibody titre) in those with sub-optimal immunity (e.g. elderly) or where antigen immunogenicity is low; but little is known about the influence of high-level training and nutritional interventions on the success of influenza vaccination in young, healthy athletes. A distinct advantage of the vaccine model (e.g. influenza) is that athletes may be keen to participate in a study where the clinical protection afforded by the vaccine is directly beneficial to them (147,406). Recognised limitations with the vaccine model include that the *ex vivo* T cell response to influenza vaccination has been more strongly related to vaccine protection than the circulating antibody titre that is typically measured (304). Also, the incorporation of repeat antigens in the influenza vaccine elicits a mixture of primary and secondary antibody responses; thus providing limited mechanistic insight (55,406). Using a novel antigen in the DTH method (e.g. keyhole limpet haemocyanin) (369); or CHS method (e.g. diphenylcyclopropenone (DPCP))(109,164) presents the opportunity to assess the influence of exercise as a stressor, and nutritional interventions on both the primary and secondary immune response. The DTH and CHS methods (Table 6) also overcome some other limitations with the vaccine method including: variable immunogenicity (e.g. hepatitis B (177)); annual changes in vaccine composition (e.g. influenza (55)); and, difficulty when comparing the circulating antibody results from different studies using in-house enzyme-linked immunosorbent assays (ELISA) or other technologies (55). Nevertheless,

Table 6. Classification and ratings of biomarkers used in immunonutrition and exercise experiments

Ratings on a continuum where: OOOOO lowest to ●●●●● highest utility. [1]Clinical relevance = considers relation to clinically meaningful outcome; [2]Scientific rating = considers validity (link to immunological mechanism(s)), reliability and diagnostic accuracy (i.e. sensitivity and specificity); [3]Practical status = considers convenience, speed, invasiveness and cost. WURSS = Wisconsin upper respiratory symptom survey; URTI = upper respiratory tract illness; Ig = immunoglobulin; DTH = delayed type hypersensitivity; KLH = keyhole limpet haemocyanin; CHS = contact hypersensitivity; DPCP = Diphenylcyclopropenone; SIgA = secretory IgA; NK = Natural Killer; CTL = Cytotoxic T Lymphocyte; CMV = Cytomegalovirus; HV = Herpes Virus; CVD = Cardiovascular Diseases; CRP = C-Reactive Protein.

Method	Clinical Relevance[1]	Scientific Rating[2]	Practical Status[3]	Overall Rating	Comments	Example Ref.
Upper Respiratory Tract Illness						
Jackson common cold questionnaire – 8 items	●●●●○	●●●●○	●●●●●	●●●●○	Symptoms derived from >1,000 individuals after nasal instillation with common cold	191
WURSS – 21 or 44 items	●●●○○	●●○○○	●●●●○	●●●○○	Considers URTI symptom impact on quality of life. Weaknesses: lengthy to complete, external validity	20
Live common cold challenge (experimental infection)	●●●●●	●●●●○	○○○○○	●●●○○	Strong clinical and scientific utility. Weaknesses: ethics, cost, medical support and quarantine	87, 88
Pathological determination of URTI	●●●●○	●●●●○	●○○○○	●●●○○	Useful partnered with symptomatology to identify infectious *vs*. non-infectious aetiology	93, 374
Physician identified URTI	●●●○○	●●○○○	●●○○○	●●○○○	Weaknesses: unstandardized; studies have questioned utility	93, 374
URTI symptom log	●●○○○	●○○○○	●●●●○	●●○○○	Weaknesses: unstandardized; preference is to use an externally valid questionnaire e.g. Jackson	20, 191
In vivo Immunity						
Ig response to vaccination (influenza, hepatitis B, pneumococcal)	●●●●○	●●●●○	●●●○○	●●●●○	Clinically relevant, beneficial to participant; hepatitis B vaccination allows study of primary and secondary Ig response; weaknesses: influenza vaccination elicits a mixture of primary and secondary Ig responses; variability in vaccine immunogenicity and Ig assays	55 147, 304, 406
DTH skin tests (e.g. KLH, multitest)	●●●●○	●●●●○	●●●○○	●●●●○	Clinically relevant; novel antigens (e.g. KLH) enable investigation of primary and secondary response. Weaknesses: relation to URTI unclear; Merieux multitest no longer available; intradermal injection and skin swellings may be uncomfortable	52, 369
CHS skin tests (e.g. DPCP)	●●●○○	●●●●○	●●●●○	●●●●○	Clinically relevant; novel antigens (e.g. DPCP) enable investigation of primary and secondary response; patches applied to skin (not intradermal); simple measurement (e.g. skin fold). Weaknesses: relation to URTI needs investigating; skin reactions may be uncomfortable	109, 164
Mucosal Immunity						
Saliva SIgA	●●●○○	●●●○○	●●●○○	●●●○○	Clinically relevant; convenient collection. Weaknesses: lack of standardisation; assays are time consuming and costly; relation to URTI in athletes is not definitive; requires individual baseline	145, 271
Tear fluid SIgA	●●●○○	●●●○○	●●●○○	●●●○○	Clinically relevant; shows promise. Weakness: only one study to date	161
Ex vivo/In vitro Immunity						
Phagocytosis and oxidative burst assays	●●○○○	●●●○○	●●●○○	●●●○○	Moderate clinical relevance; scientifically justified useful indicators for body's overall innate immune defence. Phagocytosis and oxidative burst can be measured at the same time using flow cytometric method. Weaknesses: not pathogen-specific; requires analysis of fresh samples in the same day; lack of standardisation in analysis procedure and reference values; limited to within-study comparison	9, 130

Method	Clinical Relevance[1]	Scientific Rating[2]	Practical Status[3]	Overall Rating	Comments	Example Ref.
Cytotoxicity assays (CTL, NK)	●●○○○	●●●○○	●○○○○	●●○○○	Moderate clinical relevance; scientifically justified useful indicators for assessing body's defence against viral and other intracellular infections. Weaknesses: time consuming for target cell labelling; not pathogen-specific for NK; could be pathogen-specific (CTL) but requiring use of *ex vivo* priming and re-challenge; lack of standardisation in analysis procedure and reference values; requires same-day analysis; time consuming and costly; requiring sterile culture condition; conventional methods involving use of radioisotope labelling; limited to within-study comparison	7, 267, 418
Lymphocyte proliferation	●●○○○	●●●○○	●●○○○	●●○○○	Moderate clinical relevance; scientifically justified useful indicators for cell-mediated immune response. Weaknesses: polyclonal reaction but not antigen/pathogen-specific, or could be pathogen-specific but requiring experimental infection or vaccination and *ex vivo* re-challenge; lack of standardisation in analysis procedure and reference values; requires blood samples for relatively rapid processing; requiring sterile culture condition; conventional methods involving use of radioisotope and special equipment for sample harvesting and counting; the alternative fluorescence dye tracking method requires flow cytometer and is more time-consuming; limited to within-study comparison	159
Immune Cell Trafficking and Other Markers						
White blood cell count	●●●●○	●●●○○	●●●●○	●●●○○	High clinical relevance at the presence of symptoms such as fever, localized pain, or cough & sputum representing bacterial infection, scientifically justified as mentioned in the text, sensitivity is high for bacterial infection but low for viral infection, false positive results when asymptomatic and may represent sympathetic activation, classical marker for bacterial infection. Weakness: requires blood samples for relatively rapid processing	24, 261
Left shift (band/segmented neutrophil)	●●●●○	●●●○○	●●●●○	●●●○○	High clinical relevance at the presence of symptoms such as fever, localized pain, or cough & sputum representing bacterial infection, scientifically justified as mentioned in the text, sensitivity is high for bacterial infection but low for viral infection, classical marker for bacterial infection. Weakness: requires blood samples for relatively rapid processing	182
NK cell count	●○○○○	●○○○○	●○○○○	●○○○○	Low clinical relevance, may represent sympathetic activation, limited target cells (CMV, HV infected cells and some type of cancers). Weaknesses: assays are time consuming and costly (flow cytometric analysis) requiring blood samples for relatively rapid processing	238, 252
Lymphocyte count	●●●○○	●●●○○	●●●○○	●●○○○	Moderate clinical relevance, decreased in systemic viral infection or autoimmune diseases due to chemokine mediated recruitment. Weakness: requires blood samples for relatively rapid processing	238
CD4/CD8	●●○○○	●○○○○	●○○○○	●○○○○	Low clinical relevance except for elderly individuals. CD8 previously considered to represent immune suppression but are now considered as a subpopulation of T cells with cytotoxic property. Weaknesses: assays are time consuming and costly (flow cytometric analysis) requiring blood samples for relatively rapid processing	381, 419
N/L Ratio (Neutrophil/Lymphocyte)	●●○○○	●●●○○	●●●○○	●●○○○	Moderate significance for asymptomatic healthy person as a marker of sympathetic nervous system activation, no association with immune responses but relevant to athletic condition, a potential risk factor for hypertension and type-2 diabetes. Weakness: requires blood samples for relatively rapid processing	47, 428
Cytokines production and soluble cytokine receptors	●●●○○	●●●●○	●●●○○	●●●○○	Clinically relevant specific. Weaknesses: blood draw and long processing, costly	117, 338
CRP	●●●○○	●●○○○	●●●●○	●●●○○	Clinically relevant. Established risk factor for CVD	290

determining the clinical significance of DTH and CHS responses, with specific regard to infection, is an important avenue for future research. It is possible that the strength of the cutaneous recall response e.g. to DPCP (109) could be generalised beyond skin immunity to indicate the immune system's general ability to respond to an infectious challenge. Available evidence from clinical studies supports this notion as cutaneous immune measures are impaired in individuals with acute infectious illness (26); they also track immune status and predict mortality in critically ill human immunodeficiency virus (HIV)-infected patients (113).

Mucosal immunity

Assessing immune markers in saliva and tears is of interest to exercise immunologists, particularly those working in the field environment. This is because collection of these fluids is non-invasive, convenient, practical and low cost (Table 6) but also because as many as 95% of all infections are thought to be initiated at the mucosal surfaces (46). This highlights the important role mucosal immunity plays in defence against opportunistic infections such as the common cold (46). SIgA production is the major effector function of the mucosal immune system providing a 'first line of defence'. SIgA is known to exhibit broad-spectrum antimicrobial activity against a range of viral and bacterial pathogens, through inhibition of pathogen adherence and penetration of the mucosal epithelia and by neutralising viruses within the epithelial cells during transcytosis (48).

Exercise and nutritional interventions might conceivably alter mucosal immunity at the level of local B cell antibody synthesis (e.g. via altered autonomic nervous system (ANS) activity or altered nutrient availability) (291) and/or by altering the transport of IgA (e.g. via altered ANS activity or availability of polymeric Ig receptor (69,320)). On this premise, there has been widespread attention and optimism that salivary SIgA may serve as a non-invasive biomarker of mucosal immunity and common cold risk. Research demonstrates that salivary SIgA has some utility for a monitoring application, whereby an individual's normal, healthy reference range is determined under standardised conditions and atypically low values can indicate an increased likelihood of common cold (145,271). The availability of a point-of-care tool for measuring salivary SIgA has made its monitoring in athletes possible (85), highlighting an avenue for continued research efforts. Perhaps not surprisingly, given the large individual variability, research has not convincingly demonstrated the utility of salivary SIgA as a predictive biomarker for the common cold by identifying atypically low values for an on-the-spot application (against the population reference range). Studies have shown a decrease in salivary SIgA lasting for an hour or more after prolonged exercise (146); this is in line with the 'open window hypothesis' (389,402). However, studies have also shown no change (32) or even an increase in salivary SIgA after prolonged exercise (40). Low levels of saliva SIgA during fluid and energy deficits (295) and in those deficient for vitamin D (171) were countered by additional energy intake in one study in military personnel (110) and by vitamin D supplementation in another study in athletes (170). Nevertheless, there are examples of conflicting evidence for mucosal immune responses to nutritional supplementation. Twelve weeks of bovine colostrum supplementation increased salivary SIgA ~80% in athletes in one study (96) but had no effect in another (198). The levels of salivary SIgA and other saliva antimicrobial proteins (AMP) (e.g. lysozyme) reported in the literature are highly variable: key considerations include that multiple glands contribute to saliva composition, and likely to its variable composition. There are also various potential confounders (e.g. diurnal variation (111); psychological stress (193); nutritional status (295) and gender (169)). Those wishing to measure salivary SIgA (and other AMP) in their studies should: standardise the saliva collection method (e.g. 5 min passive drool); standardise the reporting of salivary SIgA (report both SIgA concentration and secretion rate) (40,404); and control potential confounders. With the search for viable alternatives in mind, a recent study demonstrated that tear SIgA (tear is secreted only by the lacrimal gland) has potential as a non-invasive biomarker of mucosal immunity and common cold risk (161). Decreased levels of tear sIgA but no change in salivary SIgA were observed during pathology-confirmed common cold and the week before individuals reported common cold symptoms. The risk of common cold symptoms the following week increased nine-fold in those with low tear SIgA. Further advances in nanotechnology and microfluidics might, in the near future, afford the possibility for on-the-spot tear fluid measurement devices and even continuous bio-monitoring by contact lenses.

***Ex vivo/in vitro* immunity**

To assess if a nutritional intervention is effective in modulating immune response, it is necessary to use appropriate markers as measurable outcomes. A variety of markers has been identified as suitable for assessing different aspects of immune system status and functionality, and different techniques are developed to measure these markers with varied sensitivity, accuracy, feasibility, and relevance for the intended purpose. Investigators can select appropriate markers based on the specific objectives of their studies. These markers, along with the studies for which they are adopted, are usually classified as *in vivo*, *ex vivo*, and *in vitro* markers/studies. In the *in vivo* setting, both nutritional intervention and the assessment of the immune response are conducted using a living subject (human or animal). This approach best reflects the physiological response to the intervention. In the *ex vivo* setting, the intervention is administered to a living subject but the immune function analysis is conducted outside of the body using accessible biological materials. For example, assessing the ability of the immune cells isolated from the blood of study participants to respond to a stimulus which mimics pathogenic or non-pathogenic agents to which the immune cells might be exposed. The incubation is performed in the presence of a synthetic media enriched in serum that can be from an animal, typically foetal calf serum, or from a human subject. This approach, particularly when performed in the presence of a subject's serum, can provide very useful information about how the immune cells might respond when challenged *in vivo*. The *in vitro* setting for both the intervention and assessment are conducted outside of the body using either cells from the participant or cell lines. Thus the key difference between the *ex vivo* and *in vitro* studies is whether the intervention is administered to humans/animals or added to the cell culture. *In vitro* experiments alone have limited value, but

they are usually used as a screening tool to search for candidates of interest for animal or human studies, or for further investigation of the cellular and molecular mechanisms of an intervention that has shown functional efficacy *in vivo*. A brief description is provided below to introduce the most commonly used *ex vivo* and *in vitro* indices for assessing immune function in exercise and nutrition research (Table 6).

In these assays, immune cells are separated from the body (or from stock as cell lines) and maintained in culture medium. Various stimuli, e.g. mitogens, are used to activate cells to make them proliferate, synthesize and release soluble factors, directly attack target cells, or engulf the added foreign particles (microorganisms or inert substances).

Phagocytosis

Phagocytosis is a process by which specialized phagocytic cells (neutrophils and monocytes/macrophages) engulf and internalize solid matter. Phagocytosis is a key mechanism of the innate immune system to contain and kill invading pathogens, or to process them for antigen presentation to T cells, and activating the adaptive immunity. The substrates used for phagocytosis assay include bacteria, yeast, red blood cells, and inert particles. One popular method is to incubate one of these substrates labelled with fluorescence with phagocytic cells and then determine the cellular uptake of these substrates using a flow cytometer. This assay can be coupled to evaluation of oxidative burst and bacterial killing, as described below.

Oxidative (respiratory) burst

This reaction is triggered by phagocytosis or exposure to certain inflammatory mediators causing a dramatic increase in oxidative metabolism which will result in the rapid generation and release of reactive oxygen species by neutrophils and monocytes. These released toxic compounds are used to kill bacteria after phagocytosis. Oxidative burst is commonly measured either using cytochrome C reduction with a photometry method, the chemiluminescence conversion method is used by most people now, or based on changes in fluorescence properties of appropriate substrate compounds, which can be assessed by flow cytometry. The bactericidal assay adds live bacteria to a culture of phagocytes, subsequently measuring phagocytosis and the destruction of pathogens.

Cytotoxicity assay

Activity of both cytotoxic T lymphocytes (CTL) and NK cells can be assessed by a cytotoxicity assay. CTL recognizes cells on the basis of their cell-specific surface antigens. NK cells, a special type of lymphocyte, directly lyse tumour cells or virally infected cells. In the CTL activity assay, target cells can be lymphoblasts, cultured tissue cells, or tumour cells. In NK activity assay, tumour cells serve as target cells. In both assays, target cells are labelled with ^{51}Cr and then incubated with effector cells (e.g. human peripheral blood mononucleocytes (PBMC), or animal spleen cells as a source of NK cells) at different ratios. The percentage of ^{51}Cr released is calculated to represent lysis of target cells which reflects the cytotoxicity of effector cells. Flow cytometry methods are now used as a favourable non-isotope alternative to the classical ^{51}Cr method.

Lymphocyte proliferation

The proliferative response of lymphocytes is frequently used to assess cell-mediated immune response. Cell proliferation can be quantified by measuring incorporation of [^{3}H]-thymidine into DNA, or non-radioactive methods such as bromodeoxyuridine incorporation, and fluorescence dye dilution assays. Common agents used to simulate lymphocyte proliferation are the T-cell mitogens concanavalin A (Con A), phytohaemagglutinin (PHA), and anti-CD3 Ab, all of which stimulate T-cell proliferation. LPS stimulates B-cell proliferation, and pokeweed mitogen (PWM) stimulates proliferation of both T- and B-cells.

Cytokine production

Immune cells produce an array of protein mediators called cytokines, which serve as messengers to regulate immune cell activities or directly exert effector function. Cytokine quantification is helpful for assessing both general state and particular functions of immune system. Agents used to stimulate lymphocyte proliferation as mentioned above as well as some cytokines and growth factors can induce high levels of cytokine production in culture medium which can be conveniently quantified by ELISA. In addition, intracellular cytokine levels in specific cells can be assessed in a mixed cell population (without the need for purification specific cell types) using flow cytometer and a combination of stains for cell surface markers and antibodies (Ab) against the cytokine of interest.

One difficulty in interpreting data obtained from cytokine analysis is that many of them are, at times, redundant and at other times oppose function. This makes it difficult to gain insight into the "real change" in function of the immune system based on the impact of an intervention on relatively few cytokines measured. Assay costs can constrain the numbers of samples measured. Fortunately, in recent years, the emerging "omics" technologies (genomics, proteomics, and metabolomics) have provided the opportunity to obtain a more holistic and integrated view of the basal and stimulated potential for cytokine production using small aliquot of sample and more sophisticated data analysis approaches. Combined with the conventional assays, these newer approaches will provide a more comprehensive and insightful understanding of whether and how nutritional intervention impacts cytokine production and the function of the immune system in general. Furthermore, these approaches will help address and reduce the well-known intra-individual variation observed in response to exercise and nutritional interventions. The "omics" information comes from genomic and epigenetic regulations (alleles, single nucleotide polymorphism, methylation), post-transcriptional regulation (microRNA), and proteomics screening for isomeric forms of protein and post-translational modification. These can be used as tools to identify the intrinsic inter-personal differences and to design personalized interventions to achieve optimal results.

Leucocyte trafficking

Leucocytes or white blood cells, including granulocytes (neutrophils, eosinophils, and basophils), monocytes, and lymphocytes (T-cells, B-cells, NK-cells), have essential roles in host defence. Leucocyte counts have long been used as a simple

clinical marker of inflammation and/or immune function but, because there is a variety of mechanisms which also influence each other, careful consideration is necessary to understand the implication of the observed changes in the leucocyte counts in association with exercise and training.

Acute alteration of leucocyte counts by a single bout of exercise

A single bout of exercise results in changes in the circulating numbers of leucocytes. The direction of change depends on the duration and intensity of the exercise. Short-term exhaustive exercise of less than one hour induces leucocytosis mainly due to increases in the circulating numbers of neutrophils and lymphocytes including NK cells, whereas prolonged exercise beyond one hour induces a marked neutrophilia. After strenuous or prolonged exercise, circulating T-cell and NK cell counts usually go down transiently below baseline within about 30 minutes after exercise (39,310). All these changes may be attenuated by carbohydrate ingestion. Leucocytosis after high intensity running in marathon runners or long distance running (18-20 km) was attenuated by carbohydrate supplementation (188,280).

Leucocyte count in persons with different level of aerobic fitness or with obesity

As a long term effect of exercise training, recent studies demonstrated the association between fitness measurements and leucocyte counts. Maximal metabolic equivalents (METs) achieved during a treadmill exercise test were inversely associated with total leucocytes, neutrophil and basophil counts after adjustment for age, whereas body mass index (BMI) was positively associated with total leucocyte, neutrophil, lymphocyte, monocyte, and basophil counts in a cross sectional survey of non-smoking healthy men (195). The Dose-Response to Exercise in Women Aged 45–75 yr (DREW) study demonstrated an exercise-volume dependent decrease in total leucocyte and neutrophil counts after 6-month moderate intensity exercise intervention for 390 sedentary obese postmenopausal women randomly assigned to different doses of exercise (196). The decrease in the waist circumference, but not the decrease in BMI, correlated with the reduction of leucocyte and neutrophil counts in this study. During a study with a mean follow-up period of 45.6 months, obesity was associated with leucocytosis with low grade elevation of serum acute phase CRP without any recognized cause of leucocytosis other than being obese (176).

Mechanism of alterations in leucocyte trafficking

Haematopoiesis, retention, release and clearance of leucocytes need to be considered to understand leucocyte trafficking. They are known to be differentially regulated across different subsets of leucocytes. Myeloid cells are constantly produced in the bone marrow under the regulation of the local microenvironment, in which stromal cells play the central role. Upon conventional inflammatory response initiated by bacterial infection, monocyte or dendritic cell derived granulocyte macrophage colony-stimulating factor and granulocyte colony-stimulating factor accelerate proliferation of myeloid progenitors as well as their maturation. CXC chemokines, such as IL-8, produced at the site of inflammation or infection, recruit neutrophils from the bone marrow (377). Malnutrition has long been recognized as a factor that suppresses haematopoiesis leading to anaemia and pan-leucopaenia. Recently, protein malnutrition was found to promote adipogenic differentiation of bone marrow stromal cells, which affects haematopoiesis (98).

Chemokine receptor CXCR4 and the corresponding ligand CXCL12 are essential for retention and homing as well as for the clearance of leucocytes. CXCL12 is abundantly expressed in the stromal cells of bone marrow as well as in the endovasculature in various organs such as spleen, liver and lung. CXCL12 expression on bone marrow stromal cells was recently shown to be decreased by noradrenaline released from the sympathetic terminal in the bone marrow via the β adrenergic receptor. Circadian fluctuation of noradrenaline release in the bone marrow is suggested to be the major cause of circadian fluctuation of leucocyte counts, especially neutrophils (352). On the other hand, the corresponding receptor CXCR4 on lymphocyte is known to be under the control of glucocorticoid. Strenuous or exhausting exercise that induces hypothalamic pituitary adrenal axis activation is considered as the major cause of post exercise lymphopaenia (294). Interestingly, senescent neutrophils expressing CXCR4 are redeployed into the bone marrow, where they are subjected to phagocytic elimination (378). Stressful exercise, such as prolonged, exhaustive or high intensity strenuous exercise, may therefore lead to post exercise lymphopaenia.

NK cells (CD56 dim cytotoxic NK subset) and CD8 positive T cells, γ-δ T cells are major cytotoxic leucocytes in the circulation. These cytotoxic effector leucocyte subsets comprise the marginal pool by an adhesion molecule CD11a/ fractalkine CX3CR1-mediated attachment to the endothelium. Adrenaline rapidly attenuates this attachment without affecting the expression of the adhesion molecule to allow demargination and release of these leucocytes into circulation to induce adrenergic leucocytosis through β2 adrenergic stimuli (112). Adrenergic stimulus is not only delivered through bone marrow sympathetic nerve terminal, but also through catecholamines released from either sympathetic nerve terminals in various organs and adrenal medulla.

In summary, during a single bout of exercise with either peripheral sympathetic nerve activation or systemic adrenaline response during prolonged exercise when extensive lipolysis is required, neutrophils and monocytes of myeloid origin will be released from bone marrow by CXCL12 downregulation. Catecholamines would also attenuate CD11a/CXCR1 mediated attachment of marginated cytotoxic leucocytes including NK cells resulting in marked leucocytosis. Neutrophils are known to have a blood half-life of 6.5 hours, and the majority of aged neutrophils would express CXCR4 several hours after the release from bone marrow and will return to bone marrow for clearance by phagocytes. In conditions of exercise with a glucocorticoid response, lymphocytes will be redeployed to secondary lymphoid tissues by enhanced CXCR4 expression and may lead to transient lymphopaenia.
Enhanced sympathetic activity in obese individuals seems to play a central role in the observed leukocytosis similar to the observed elevated blood pressure. The obesity-associated chronic inflammation, through increased leptin production,

enhances sympathetic activity and therefore leukocytosis (156). Therefore, attenuation of obesity-related leucocytosis after sessions of exercise training may either be the result of decreased sympathetic activity through weight reduction or because of anti-inflammatory effect of exercise leading to reduced leptin release or both.

Clinical implication and limitations

Impaired adhesion or excess retention of leucocytes both compromise self-defence as demonstrated in primary immuno-deficiency patients with leucocyte adhesion-deficiency (LAD) syndrome (293). LAD is caused by the mutations of the gene encoding for the β-2 integrin CD18, which is required for firm adhesion of leucocytes to endothelium before transmigration. LAD patients demonstrate marked leucocytosis with high incidence of gingivitis, periodontitis, cutaneous infections without pus formation, life-threatening bacterial infections and delayed wound healing.

The clinical implication of altered leucocyte trafficking by exercise is that, during sympathetic activation, even though the circulating leucocyte number is high, it is possible that immune or inflammatory reaction through leucocytes may be blunted because of attenuation of adhesion through β adrenergic stimuli. This may avoid both systemic and local immune responses and inflammation that affects "fight or flight" actions. However, a similar situation may happen when metabolic demand is high and after prolonged exercise or in obesity with sympathetic activation. Therefore, leucocytosis in such situations may be considered as a new "open window". Previously the term "open window" referred to the occurrence of lymphopaenia or decreased NK cells after strenuous exercise due to retention in secondary lymphoid organs and marginal pool. However, a mild and transient retention to their site of action may not confer impaired immunity. This might be explained by the fact that NK cells have a limited spectrum of cytotoxicity, which is strictly determined by the expression of variant MHC-I (major histocompatibility antigen 1) or loss of MHC-1 due to either viral infection such as CMV or herpes simplex virus or in some tumour cells (70).

Carbohydrate ingestion known to attenuate leucocytosis is likely to be mediated by attenuation of sympathetic drive by reducing the energy demand, which may be an effective way to close the open window, as previously recognized. On the other hand, reduced energy intake as often observed in female athletes' triad or relative energy deficiency in sports, may affect haematopoiesis in starvation or malnutrition (265). Although there is insufficient clinical information related to leucocyte trafficking, it is likely that serious cases may present leucopaenia as well as anaemia, leading to impaired immunity when haematopoiesis is limited. At the earlier phase of reduced energy intake, sympathetic driven lipolysis may attenuate leucocyte adhesion, and this may compromise neutrophil defence as mentioned above.

Conflict of Interest

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Developing a multi-component immune model for evaluating the risk of respiratory illness in athletes

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ABSTRACT

Clinical and laboratory identification of the underlying risk of respiratory illness in athletes has proved problematic. The aim of this study was to determine whether clinical data, combined with immune responses to standardised exercise protocols and genetic cytokine polymorphism status, could identify the risk of respiratory illness (symptoms) in a cohort of highly-trained athletes. Male endurance athletes (n=16; VO_2max 66.5 ± 5.1 mL.kg^{-1}.min^{-1}) underwent a clinical evaluation of known risk factors by a physician and comprehensive laboratory analysis of immune responses both at rest and after two cycling ergometer tests: 60 min at 65% VO_2max (LONG); and 6 x 3 min intervals at 90% VO_2max (INTENSE). Blood tests were performed to determine Epstein Barr virus (EBV) status and DNA was genotyped for a panel of cytokine gene polymorphisms. Saliva was collected for measurement of IgA and detection of EBV DNA. Athletes were then followed for 9 months for self-reported episodes of respiratory illness, with confirmation of the underlying cause by a sports physician. There were no associations with risk of respiratory illness identified for any parameter assessed in the clinical evaluations. The laboratory parameters associated with an increased risk of respiratory illnesses in highly-trained athletes were cytokine gene polymorphisms for the high expression of IL-6 and IFN-ɣ; expression of EBV-DNA in saliva; and low levels of salivary IgA concentration. A genetic risk score was developed for the cumulative number of minor alleles for the cytokines evaluated. Athletes prone to recurrent respiratory illness were more likely to have immune disturbances that allow viral reactivation, and a genetic predisposition to pro-inflammatory cytokine responses to intense exercise.

KEYWORDS: exercise, athletes, respiratory infections, inflammation

INTRODUCTION

Upper respiratory illness is the most common reason for non-injury related presentation in sports medicine, accounting for 35-65% of illness presentations in elite athletes in training and competition (12, 14, 41). Recurrent respiratory illness can have a negative impact on the health and performance of athletes undertaking high levels of strenuous exercise, and interferes with training and ability to compete in international competitions in up to 10% of athletes (2, 48, 52). The majority of athletes have a similar incidence of upper respiratory illness to the general population (15), but a small proportion (5-7%) experience recurrent episodes of upper respiratory symptoms (URS) at significantly higher rates. The incidence of URS increases during periods of intense training, in association with increases in training load (32, 38) and around competitions (41, 44, 55). Identifying athletes at risk of recurrent URS allows adoption of preventative strategies based on relevant clinical, training and lifestyle modifications.

The common symptoms associated with upper respiratory illness include a sore throat, headache, fatigue, runny nose and/or watery eyes. The cause of URS in athletes is often unknown as pathology testing is rarely undertaken and physicians may not be available to undertake comprehensive clinical assessments in research studies. Pathology investigations have identified infections as a cause of the symptoms in only 30-40% of high-performance athletes studied (6, 53).A higher frequency has been observed in recreational athletes (28). Bacterial respiratory infections are uncommon in elite athletes (25) and the majority of identified infections are common res-

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piratory viruses found in the general population (6, 15, 29, 49, 53). While transient exercise-induced immune suppression can increase susceptibility to infection (58), not all episodes of URS have an infective aetiology (6, 49) and susceptibility is influenced by other lifestyle and environmental factors (29, 38, 55).

Non-infective inflammatory causes of URS include allergic responses to aeroallergens, asthma, and trauma to respiratory epithelial membranes, particularly in athletes who experience drying of the airways related to increased expired ventilation or cold air exposure (27). Undiagnosed or inappropriately treated asthma and/or allergy are common findings in clinical assessments of elite athletes experiencing recurrent URS (6, 49). Inhaled allergens can affect approximately 40% of athletes (6, 49) with 20-40% having rhinoconjunctivitis (6, 35, 56) that responds to treatment with topical medications (7, 34). Psychological stress, sleep disturbances and dietary deficiencies have also been associated with increased URS (30, 38).

There has been extensive examination of changes in immune parameters with exercise, but only a limited number have included assessment of URS in the study design (27, 29, 33). Many of the conclusions that both high levels of exercise intensity and sedentary behaviours are associated with susceptibility to URS have been inferred from the changes in immune parameters. These changes have the potential to leave a person at increased risk of infection.

The measurement of secretory IgA in saliva has shown consistent associations with URS in athletic populations (58). Secretory IgA is an important component of protection against infections at mucosal surfaces, together with integrity of the epithelial barrier and regulatory T-lymphoid cells. Low levels of salivary IgA, decreased IgA secretion rates, and a decline in salivary IgA concentration over a training period have been associated with a higher risk of URS (25, 26, 33, 44). Moderate exercise, as opposed to intense exercise, can increase salivary IgA and has been used to modify training in athletes at risk of or with a history of recurrent URS (23, 37). A recent study of tear fluid secretory IgA (SIgA) has also shown lower levels of SIgA and excretion rates in tears but not in saliva with an increase in the risk of upper respiratory tract infections in athletes (29).

Illness-prone athletes can have altered/adverse cytokine responses to standardised intense treadmill exercise protocols in comparison with healthy athletes (11) and a genetic predisposition to pro-inflammatory cytokine responses (5). The cytokine responses to the exercise included lower resting levels of IL-8, IL-10 and IL-1ra in illness-prone athletes, lower levels of IL-10 and IL-1ra but higher levels of IL-6 post exercise, collectively indicating impaired inflammatory cytokine regulation in illness-prone athletes (11). A study of cytokine gene polymorphisms identified a trend for a high-expression genotype for IL-6 in illness-prone athletes and a high-expression genotype for IL-2 associated with a decreased likelihood of recurrent URS in a cohort of 170 elite athletes (5). Two studies differ on the impact of IL-10 genotypes on risk of URS, with one showing no impact (5) and the other indicating an increased risk with the high-expression IL-10 genotype (61).

Studies have examined the effectiveness of various biomarkers to identify athletes at risk of URS, based on the premise that transient immune alterations after exercise provide a window of opportunity for infections. While fitter adults are less likely to experience URS than sedentary individuals (4), this paradigm does not hold true for the 7-10% of elite athletes who experience recurrent URS (15, 24, 30). There appears to be a threshold of training load that puts athletes at increased risk of URS. Longitudinal studies have identified the impacts of intense training (24, 39) on decreased concentration or excretion rates of salivary IgA and an increased risk of URS (58).

Epstein Barr virus (EBV) infection is a common presentation in elite athletes and viral reactivation of EBV is a common finding in research settings (22-50%) in athletes experiencing recurrent URS (8, 60). Expression of EBV DNA in saliva is associated with a prior reduction in salivary IgA levels and subsequent appearance of URS (28, 60). IgA plays a major role in controlling viral reactivation and low levels prior to the appearance of EBV viral DNA indicate that salivary IgA could be used as a surrogate marker for increased risk of viral reactivation and the associated inflammation that occurs from the immune response to the viral particles shed in the respiratory tract (20). Midkine is a constituent of the mucosal innate immune system with potent bactericidal and fungicidal properties and plays a role in inflammatory processes at mucosal surfaces (19). Traditional biomarkers of innate immunity, such as lactoferrin and lysozyme, have also shown associations with URS in athletes (59). Midkine concentration has not been assessed previously in relation to responses to intense exercise and was included in this study as a potential new marker of risk for URS in elite athletes.

The aim of this study was to determine whether clinical data, combined with immune responses to standardised exercise protocols and genetic cytokine polymorphism status, can be used to identify the risk of respiratory illness (symptoms) and associated fatigue in elite athletes. This study assessed selected clinical and laboratory parameters, known to be associated with URS, in a prospective study to identify athletes at risk of a high incidence of URS. The study design included a clinical assessment for known causes of respiratory infections and airway inflammation, laboratory tests for genetic predisposition to pro-inflammatory responses and EBV status, and immune responses to short-intense and longer-endurance exercise protocols. The study involved highly-trained male cyclists who were monitored daily for nine months and then classified as illness-prone or healthy based on their reported episodes of URS during the study period. The clinical and laboratory parameters were then assessed for their effectiveness in a predictive model.

MATERIALS AND METHODS

Study Design

The study examined clinical and laboratory measures associated with highly-trained endurance athletes who experience recurrent episodes of upper respiratory tract illness due to infection and/or inflammation.

Prior to commencement of the investigations each athlete underwent a comprehensive clinical examination with a focus on a history of known causes of airway infection and inflammation, including asthma, allergy, and common respiratory infections. A full blood count (FBC) was performed prior to undertaking performance testing to exclude underlying infections and potential exclusion illnesses. Blood serology tests were performed to determine Epstein Barr virus (EBV) status and DNA was examined for cytokine gene polymorphisms. Saliva was collected prior to the VO_2max test to exclude any subject with IgA-deficiency.

Sixteen endurance-trained male cyclists were recruited to this study and prospectively followed for 9 months using a web-based daily reporting of training and illness symptoms. The type, severity and duration of illness were quantified using the AIS Athlete Illness Questionnaire (17). Each episode of illness was followed up by a physician and included completion of the Common Cold Questionnaire (46)

Each athlete completed an initial performance assessment including a VO_2max test and two subsequent cycle ergometer tests, with each test at least 7 days apart, to assess a multi-component immunological response to the exercise protocols. The VO_2max test was performed prior to commencing the study and the Long and Intense exercise tests were completed in a randomised order in the first two weeks of the study. These included plasma cytokines (IL-1ra, IL-4, IL-6, IL-8, IL-10, IL-17a, interferon-γ (IFN-γ)), C-reactive protein (CRP), salivary IgA and salivary EBV-DNA at several time points before and after the exercise tests (pre-exercise, and immediately, 1hr and 24 hr post-exercise). Saliva and blood samples were processed for storage under appropriate conditions for analysis as single batches at the conclusion of the study to reduce assay variability.

Exercise Testing - VO_2max test

At the initial visit, each athlete completed a VO_2max test. The maximal aerobic capacity (VO_2max) of each athlete was assessed by an incremental exercise test to volitional exhaustion on a Lode Excalibur cycle ergometer (Load B.V. Groningen, The Netherlands) and open-circuit indirect calorimetry system (Australian Institute of Sport, Bruce, ACT, Australia) as described previously (11). Athletes commenced at 100W with an increase of 25W every 3 min until volitional exhaustion. VO_2, heart rate and blood lactate were monitored throughout the test by standard techniques.

Exercise Protocols – Long and Intense

Each athlete completed two cycling ergometer tests in a randomised and counterbalanced order, with tests separated by a minimum of 7 days. Testing was conducted between 8-10am. Exercise testing was only undertaken if athletes were free of symptoms of illness on the scheduled day of testing and in the previous 3 days. The test protocols were: (i) 60 min at 65% VO_2 max (LONG); and (ii) 6 x 3 min intervals at 90% VO_2 max with 90 seconds of active recovery between each repetition (INTENSE). Athletes completed a 5 min warm-up on the cycle ergometer involving 3 min at a self-selected power output (range 100-150 W) then 4 x 15 sec intervals at the power output of the designated exercise intensity followed by 15 sec of self-selected active recovery. Heart rate was recorded continuously during the trial, and subjects assessed their effort using the Borg Scale 1-10 rating of perceived exertion. Whole blood lactate concentration was monitored as a measure of exertion using the Lactate Pro analyser (Arkray KDK, Japan) with 5 µl blood drawn from the earlobe or fingertip.

Laboratory Methodology

Blood Collection

Blood was collected prior to the VO_2max tests and 24 h after the exercise protocols from a superficial fore arm vein by standard venepuncture techniques. An intravenous cannula was inserted prior to commencement of each cycle ergometer exercise test to allow multiple timed blood collections, prior to and immediately after and at 1 h post exercise. Samples were collected into K_3EDTA and clot activator serum separation tubes (Greiner Bio-one, Frickenhausen, Germany).

Full Blood Count

A full blood count (FBC), including a white blood cell (WBC) differential for enumeration of neutrophil, lymphocyte and monocyte, basophil and eosinophil populations, was performed on whole blood samples within one hour of sample collection using a Sysmex XT-2000i Counter (Sysmex Corporation, Japan).

EBV serology

Serum IgM antibodies to EBV viral capsid antigen and IgG antibodies to EBV nuclear antigen were measured as previously described (8) with commercial enzyme-linked immunsorbent assay (ELISA) kits (panbio; Inverness Medical Innovations, Sinnamon Park, QLD, Australia) using a BEP2000 Advance Analyser (Siemens, Munich, Germany). All samples were analysed in a single batch to avoid inter-assay variation.

C-Reactive Protein

Serum CRP concentrations were determined using an Immulite 1000 solid phase chemiluminescent immunometric assay system (Siemens Healthcare Diagnostics, Flanders, NJ, USA) and commercially available assay kits (Diagnostics Products Corporation, CA, USA). All samples were analysed in a single batch to avoid inter-assay variation. The population reference range for serum CRP concentration was <3 mg/L.

Cytokine Concentrations

Blood samples were collected directly into K_3EDTA tubes (Greiner Bio-one; Frickenhausen, Germany) and plasma separated by centrifugation at 800g for 5 min and stored frozen at –80 °C until analysed. Plasma concentrations of each cytokine were determined simultaneously using a Bio-Plex Suspension Array System (Bio-Rad Laboratories Pty Ltd; Hercules, CA, USA) and custom manufactured Multiplex Cytokine Kits (Bio-Rad Laboratories Pty Ltd; Hercules, CA, USA) as previously described (11). The instrument was standardised with Bio-Plex Pro Human Cytokine Standard 27-Plex Group 1, Lot number 50295100 (Bio-Rad Laboratories Pty Ltd; Hercules, CA, USA).

Cytokine Polymorphisms

Nucleic acids were extracted from whole blood cells collected in K_3EDTA tubes using the QIAamp Blood Mini Kit (QIA-

GEN GmbH, Hilden, Germany). Extracted RNA was stored in Qiagen RB Sample Tubes (QIAGEN GmbH, Hilden, Germany) at -80ºC until assayed as a single batch. Assays were completed in accordance with manufacturer's guidelines using a 7500 Real Time PCR System (PE Applied Biosystems, Foster City, USA) as previously described (5). Automatic classification of samples as homozygous (for either allele) or heterozygous was undertaken using the SDS 7500 System Software Version 1.4 (PE Applied Biosystems). The cytokine polymorphisms assessed are listed in Table 8. The classification of each polymorphism was determined from the NCBI dsSNP database (available atwww.ncbi.hlm.nih.gov/SNP/).

Midkine

Midkine concentrations were measured using a commercial enzyme-linked immunsorbent assay (EBV) (Cellmid Limited, Sydney, Australia).

Saliva Collection

Saliva samples were collected passively using four commercial eye spear swabs (CoreSurgical, UK) 10 min prior to the VO_2max test, immediately prior to each exercise test, immediately after completion of each test, and at the 1 h and 24 h recovery time points. The athletes were not fasted. The eye spear swabs have been confirmed as a suitable collection method for analysis of salivary IgA (54).

Salivary IgA Concentration

The concentration of IgA was measured in each saliva sample by an in-house ELISA method as described previously (22). The between-run coefficient of variation for the internal control was 11%.

Salivary EBV-DNA

EBV viral excretion in saliva was detected using a quantitative real-time polymerase chain reaction assay. DNA was isolated from saliva samples using a QIAamp DNA Mini Kit (QIAGEN, GmbH, Hilden, Germany). Commercially available EBV-specific primers and probes (Qubit dsNA BR Assay kit, Invitrogen, Carlsbad, California, USA) were used in the real time PCR to amplify a region of the BALF5 gene, as described previously (8), using a Viia-7 rtPCR System (PE Applied Biosystems, Foster City, USA) detection system.

Statistical Analysis

All statistical analyses were performed using SAS v9.4 (SAS Institute, Cary, North Carolina, USA). Given the multiple potential outcomes measured, the Bonferroni method of adjusting for multiple comparisons was adopted. The significance level was set at $p<0.01$.

Differences between the illness-prone and healthy athletes in age, weight, fitness level, clinical history, and pre-exercise salivary IgA concentration were assessed using 2-sample t-tests or Pearson chi-square tests. Salivary IgA data were summarised at each time point using medians (with 95% confidence intervals). Within-subject differences in median salivary IgA were assessed using the sign-rank test.

For each of the cytokine SNPs the major/minor alleles in a Caucasian population were chosen using the NCBI dbSNP database (http/www/ncbi.nlm.nih.gov/SNP/), as the participants were Caucasian. The NCBI database for IFN-γ has T as the major allele in Caucasian populations. In both this study and a previous study (5) the A allele appears to be the major IFN-γ SNP in the Australian population. The impact of coding the major and minor allele for IFN-γ was assessed both ways for this study. The impact of coding A as the major allele would lead to increased inflammation for the (minor) T allele. The classification was also checked both ways in the genetic risk score analysis.

The distribution of each cytokine SNP was assessed for Hardy-Weinberg equilibrium prior to analysis. Genotypes and their association with the dichotomous outcome (illness prone or healthy, based on the number of URS during the study period) were analysed using two methods. First, individual genotyped SNPs were coded as 0, 1, or 2, representing a subject's dosage (number of copies) of the minor allele. Association with the outcome was analysed with a chi-square test of general association. Secondly, a composite genetic risk score (GRS) was generated representing the total number of minor alleles across all candidate cytokine SNPs in each individual. In this case association with the outcome was analysed using a t-test for the difference in mean genetic risk score.

As the dataset sample size was small, exact logistic regression was used to analyse the outcome against each SNP under an additive model. Estimates showing the odds ratio for the outcome with 95% confidence intervals are presented (Table 10). P-values from the exact tests are presented. As outlined above, given the number of cytokines tested, we altered the threshold for significance to $p<0.01$.

The distribution of concentrations of salivary IgA (Figure 2) and midkine (Figure 3) are presented as box and whisker plots at each time point for the Intense and Long exercise protocols. The bars in the box plot represents the 25th, 50th, 75th percentiles, the circle within the boxes (joined by dashed lines) represents the mean concentration. Circles outside the box illustrate points that exceed 1.5 times the interquartile range above the 75th percentile.

RESULTS

Study cohort

Complete clinical and laboratory data sets were obtained from 16 male athletes. The athletes were triathletes (n=4), cyclists (n=11) and a cross country skier (n=1) whose training included long distance cycling. Eight athletes competed at national or international level and the other eight at state or club level. The number of clinician-verified URS episodes during the study was used to classify the subjects as illness prone (>3 episodes). Only 4 subjects met the definition for illness-prone, which has limited the power to detect differences between the illness-prone and healthy athletes.

The physical characteristics of the study cohort were: age 32.5 ± 8.1 y; body mass 73.9 ± 7.9 kg; VO_2 max 4.9 ± 0.6 L.min^{-1}; VO_2max 66.5 ± 5.1 mL.kg^{-1}.min^{-1}; peak power 411 ± 46 W; 5.6 ± 0.5 W.kg^{-1}; mean ± SD. The mean duration of training

Table 1. Demographics of the athletes in the illness-prone (n=4) and healthy (n=12) athlete groupings and comparison between groups (mean (SD)).

Variable	Illness-prone (n=4)	Healthy (n=12)	*p*-value
Age (years)	28.6 (7.9)	33.8 (8.1)	0.28
Body mass (kg)	70.6 (6.0)	74.5 (8.3)	0.41
VO_2max			
(L/min)	5.0 (0.3)	4.9 (0.6)	0.67
(ml/kg/min)	69.6 (3.6)	65.5 (5.2)	0.12
Peak Power			
(W)	431 (38)	404 (48)	0.32
(W/kg)	5.5 (0.5)	6.0 (0.5)	0.10

for the group was 14 ± 5 h per week. There were no differences between the illness-prone and healthy athletes for age, weight, or fitness level determined by VO_2max performance (Table 1). After adjustment for body mass there was a trend for illness-prone athletes to have a higher relative VO_2 max (ml.kg/min) (p=0.12) and relative peak power (W/kg) (p=0.10) than the healthy athletes (Table 1).

Clinical History

Assessment of the clinical history and clinical interview data was unremarkable (Table 2). The results of the full blood count testing indicated the subjects were clinically healthy and showed no signs of infections or inflammation at the time of exercise testing. Six athletes (38%) had a history of asthma but only one athlete was currently being treated for asthma. Eight athletes (50%) had a history of allergy, with five having allergic rhino-conjunctivitis and 3 recording other allergies. There was no difference in the distribution of a history of any allergy (p=0.25), allergic rhino-conjunctivitis (p=0.12), asthma (p=0.55), or a combined history of asthma and/or allergy (p=0.07) between the illness-prone and healthy athletes (Table 2).

Upper Respiratory Tract Symptom Episodes

The 16 athletes had evenly distributed episodes of upper respiratory tract symptoms (URS) during the 9 month prospective study (southern hemisphere spring, summer, autumn). There were no episodes in 3 athletes (19%); 9 athletes had 1-2 episodes (56%); and 4 athletes had 4-5 episodes (25%). On average the symptoms lasted 4 days during each episode. There was no significant difference in the distribution of the number of URTI episodes reported in the previous 12 months (p=0.25) between the illness-prone and healthy athletes (Table 2). The self-reported history of URTI in the 12 months prior to the study did not match the number of URS episodes reported during the 9-month study period (Table 2).

Table 2. Reported episodes of URS during the study by each athlete, their clinical history of URTI, asthma and allergy and EBV serology status at commencement of the study, and detection of EBV-DNA in saliva samples collected during the exercise protocols. Blank spaces indicate a negative result. RC indicates the allergy was rhino-conjunctivitis. URS upper respiratory symptom, URTI upper respiratory tract illness, EBV Epstein Barr virus, DNA deoxyribonucleic acid, PRE pre-exercise, immediately POST and 24hr post-exercise. No sample was collected 24hr post Intense protocol for ID #9.

ID	Number of URS episodes during study	History of URTI in prior 12mth	History of Asthma	History of Allergy	EBV Serology Status	EBV-DNA in saliva	EBV-DNA in saliva LONG Protocol			EBV-DNA in saliva INTENSE Protocol		
						PRE VO_2 max test	PRE test	POST test	24HR Post	PRE test	POST test	24HR Post
1	0	3			Positive	Positive	Positive	Positive				
2	0	2		Yes/ RC	Positive					Positive	Positive	Positive
3	0	1	Yes		Positive							
4	1	1		Yes	Positive							
5	1	3	Current	Yes/ RC	Positive					Positive	Positive	
6	1	2		Yes/ RC	Positive		Positive	Positive				
7	1	1		Yes/ RC	Positive		Positive		Positive		Positive	
8	2	3	Yes	Yes/ RC	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
9	2	2			Positive							no data
10	2	1	Yes		Positive	Positive	Positive					
11	2	0			Positive	Positive	Positive	Positive	Positive			Positive
12	2	2	Yes	Yes	Positive	Positive				Positive	Positive	
13	4	15			Positive		Positive					
14	4	2			Positive	Positive	Positive	Positive	Positive			
15	4	2			Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
16	5	0	Yes	Yes	Negative							

Table 3. Number of URS episodes reported by the 15 EBV-seropositive athletes and the number and percentage of saliva samples with EBV-DNA detected in these athletes. No subject reported 3 URS episodes during the study.

Number of URS episodes during the study in the 15 EBV seropositive athletes	Number of athletes	Number of Pre-exercise saliva samples with EBV-DNA detected	Percentage of Pre-exercise samples with EBV-DNA detected	Number of samples from all exercise tests with EBV-DNA detected	Percentage of samples from all exercise tests with EBV-DNA detected
0	3	3/9	33%	6/21	29%
1	4	3/12	25%	7/28	25%
2	5	8/15	53%	17/34	50%
4	3	6/9	67%	12/21	57%

EBV Serology

All athletes were negative for EBV IgM serology at the start of the study, indicating no athlete had current infectious mononucleosis (glandular fever). Based on the IgG serology, 15 athletes were seropositive for prior EBV infection (94%). One athlete (ID#16) was seronegative, had no EBV-DNA detected in any saliva sample, but notably the highest incidence of URS during the study (Table 2).

Table 4. The pre-exercise test (PRE) salivary (Sal) IgA concentrations (mg/L) and mean of the three pre-exercise test results for each athlete compared to the number of reported episodes of URS and the predictive Risk category of the salivary IgA concentration. Samples were classified as Higher Risk (H) of URS for IgA <40mg/L, Lower Risk (L) for IgA > 60mg/L and Moderate Risk (M) for IgA between 40-60 mg/L for the initial VO_2max test and for the average of the three pre-exercise test (3 x PRE) saliva samples.

Subject ID	URS Episodes in study	VO_2max PRE Sal IgA	Intense PRE Sal IgA	Long PRE Sal IgA	Mean of 3 x PRE Sal IgA	Risk for VO_2max PRE	Risk for Mean of 3 x PRE
1	0	58	33	26	39	M	H
2	0	94	77	64	78	L	L
3	0	67	22	50	46	L	M
4	1	107	58	48	71	L	L
5	1	62	32	90	61	L	L
6	1	48	43	67	53	M	M
7	1	41	64	68	41	M	M
8	2	50	42	32	41	M	M
9	2	38	40	44	41	H	M
10	2	102	60	100	87	L	L
11	2	127	72	21	73	L	L
12	2	31	48	59	46	H	M
13	4	38	35	24	32	H	H
14	4	62	53	39	51	L	M
15	4	135	71	80	95	L	L
16	5	37	28	31	32	H	H
Mean±SD		69 ± 34	49 ± 17	53 ± 24	55 ± 20		

Table 5: The distribution and median concentrations of the average of the three pre-exercise salivary IgA concentration (mg/L) in the illness-prone and healthy athletes and the differences between the groups.

Pre-exercise salivary IgA concentration	Class	Illness-prone (n=4)	Healthy (n=12)	Total (n=16)	Difference *p value*
Lower Risk	>40mg/L	2 (15%)	11 (85%)	13 (81%)	0.06
Higher Risk	<40mg/L	2 (67%)	1 (33%)	3 (9%)	
Median IgA	mg/L	42	55		0.44
Min, Max	mg/L	32, 95	39, 87		

Table 6. Salivary IgA concentrations (mg/L) and 95% confidence intervals (CI) for all athletes undertaking the Intense and Long Exercise Protocols at each collection time

Saliva Collection Time	Intense Protocol Salivary IgA median (95% CI)	Long Protocol Salivary IgA median (95% CI)
Pre-exercise	45 (35, 64)	49 (32, 69)
Post-exercise	53 (38, 68)	54 (46, 64)
1 h Post	43 (33, 55)	45 (37, 78)
24 h Post	39 (28, 63)	38 (27, 56)

Table 7: Change in salivary IgA concentration (mg/L) both between- and within-subject across the time points in the Intense and Long Exercise Protocols. The sign of the change indicates the direction of median change in percentage concentration: positive is an increase; negative is a decrease.

Difference	Intense Protocol median (95% CI)	Sign Rank *p value*	Long Protocol median (95% CI)	Sign Rank *p value*
Between-subject				
Pre - Post	+0.3 (-10, +22)	0.72	-0.5 (-16, +15)	0.94
Post – 1 h	-6.9 (-21, +11)	0.21	-6.8 (-12, +25)	1.00
Post – 24 h	-5.6 (-28, -0.4)	0.03	-12 (-20, +24)	0.60
24 h - Pre	-2.1 (-19, +3)	0.42	-4.2 (-12, +12)	0.74
Within-subject				
Pre - Post	+0.2 (-20, +43)	0.56	-0.8 (-25, +61)	0.53
Post – 1 Hour	-13.2 (-28, +47)	0.56	-18 (-26, +46)	0.90
Post – 24 Hour	-9.4 (-50, -0.6)	0.05	-27 (-35, +37)	0.63
24 Hour - Pre	-4.1 (-37, +9)	0.42	-9.6 (-27, +27)	0.78

EBV viral DNA

EBV-DNA was detected more frequently in saliva samples prior to the VO_2max test, and also pre and post the two exercise protocols in athletes with a higher number of episodes of URS during the study (Table 2). The percentage of saliva samples positive for EBV-DNA in the three PRE-exercise saliva samples tended to increase with incidence of URS (p=0.14) from 33% to 67% (Table 3). Saliva samples collected immediately post and at 24h post the exercise protocols were tested for EBV-DNA (Table 3) and showed the same trend of higher detection rates in athletes with a higher number of episodes of URS (p=0.06), increasing from 24% to 52% (Table 3).

Salivary IgA

All athletes had detectable levels of IgA in all saliva samples, confirming there were no IgA-deficient subjects. Using previously established URS risk cut-off levels for swimmers (26) the athletes were classified for each saliva sample as 'Higher Risk' if salivary IgA was <40 $mg.L^{-1}$; 'Moderate Risk'if between 40-60 $mg.L^{-1}$; or at a 'Lower Risk'if >60 $mg.L^{-1}$ (Table 4). Low levels of salivary IgA (<40 $mg.L^{-1}$) were more common in the PRE VO_2max test samples for athletes reporting a higher number of URS episodes during the study (Table 4). The average of the three pre-exercise test salivary IgA concentrations was used to estimate a typical within-subject salivary IgA resting level as this takes into account the large variability within-subjects (13). The distribution of the average of the three resting pre-exercise salivary IgA levels approached significance (p=0.06) with a higher proportion of lower levels of salivary IgA (<40mg/L) in the illness-prone athletes (Table 5). The differences in the median concentrations of the average pre-exercise salivary IgA between the illness-prone and healthy groups was lower in the illness-prone group (Table 5) but not significantly different to the healthy athlete group (p=0.44).

Salivary IgA response to exercise

Salivary IgA responses to the Intense and Long exercise protocols showed variability between the athletes. To normalise the response the changes were expressed as a percentage change relative to the resting pre-exercise level for each athlete (Figure 1). The median changes in the salivary IgA concentrations for all athletes (Table 6, Figure 2), the percentage changes between time points for all athletes (Table 7) and the within-person difference (change) in median IgA levels (Table 7) immediately post exercise were not

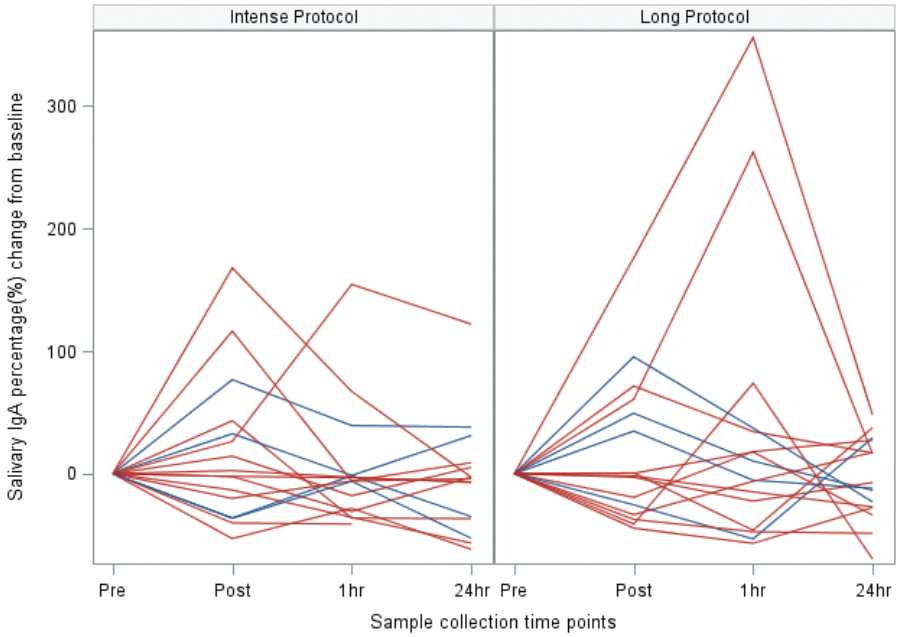

Figure 1: Salivary IgA responses to Long and Intense exercise protocols for each athlete expressed as a percentage change from the resting pre-exercise level for individual athletes. Individuals with more than 3 URS episodes are coded as blue.

significantly different from the baseline pre-exercise levels for both protocols. Differences in median salivary IgA concentrations immediately post to 1h post exercise were not significant for either protocol in any measure (Table 7).

There was evidence of a significant difference at 24 h after the Intense protocol compared to the immediate post (median difference of 8 mg.L^{-1}, p=0.03) although this did not remain significant when adjusted for multiple comparisons using the Bonferroni method (Table 7). The percentage change in salivary IgA levels for individual athletes from immediate post to 24 h post in the Intense protocol showed the same trend (p=0.05; median change of 9%) although this did not reach our significance threshold of 0.01.There were no significant differences between the 24h post and the pre-exercise test level for either protocol using any of the measures (Tables 7).

CRP and Cytokine Concentrations

There were no elevated CRP levels above the population reference range for any pre-exercise test blood sample. The CRP levels did not alter significantly during either exercise protocol. The pre-exercise test CRP levels did not correlate with the number of episodes of URS. Most of the cytokines analysed using the Multiplex technology were either not detected (IFN-γ) in any sample or detected in only a subset of the athletes (IL-1ra, IL-10, IL-4, IL-6). All athletes had detectable levels of IL-17a and IL-8 on most occasions. As a result there was insufficient power to show any statistical patterns in the cytokine responses to exercise.

Cytokine Genotype SNPs

The distribution of each cytokine SNP polymorphism in the study cohort is provided in Table 8. The exact logistic regression showed there was no clear association between the number of minor alleles for individual SNPs and having three or more URS episodes during the study (Table 9). The exception was a trend for an association with IL-6 (p=0.06) and IFN-γ (p=0.01). The genetic risk score indicates a potential for an accumulative effect of the number of minor alleles (p=0.03) although this did not meet our significance level of p=0.01.

The odds ratios (OR) for being illness-prone are provided for each SNP in Table 10. There is some evidence of a cumulative effect of increasing genetic risk score on the odds of three or more illness bouts during the study period (OR=0.49; 95% CI: 0.15,0.98; p=0.04) but this was not significant at the 1% threshold. The possible association with IFN-γ minor alleles (p=0.05) was also not significant at the 1% level.

Midkine response to exercise

There was a significant increase in midkine concentrations immediately post exercise for both exercise protocols (Table 11, Figure 3). The Midkine concentrations fell below pre-exercise levels at 1 h post but returned to pre-exercise concentrations by 24 h post-exercise.

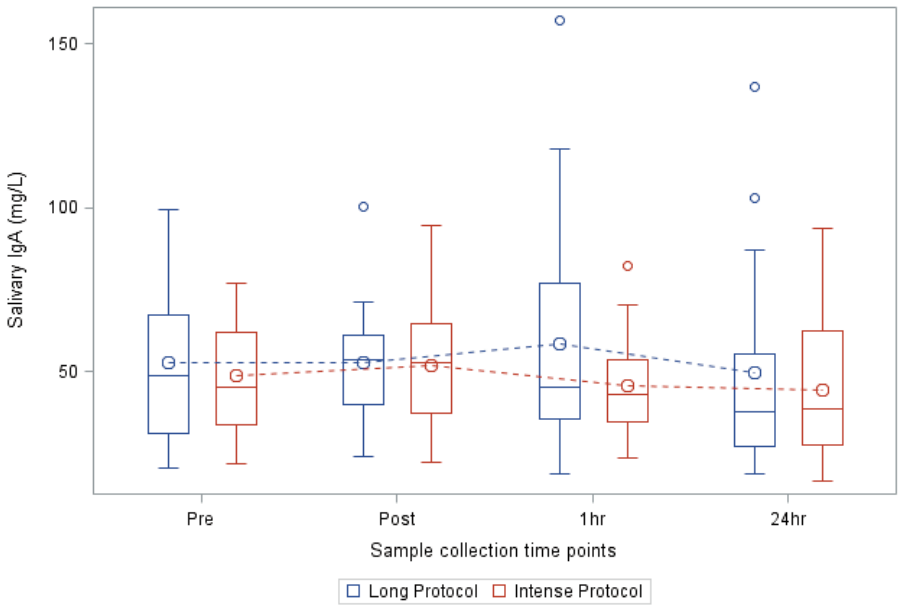

Figure 2: Distribution (median and quartiles in box-plots, mean in circles) of salivary IgA (mg/L) at each time point for the Long (Blue) and Intense (Red) exercise protocols.

DISCUSSION

A more effective means of identifying the risk of illness would assist clinicians in

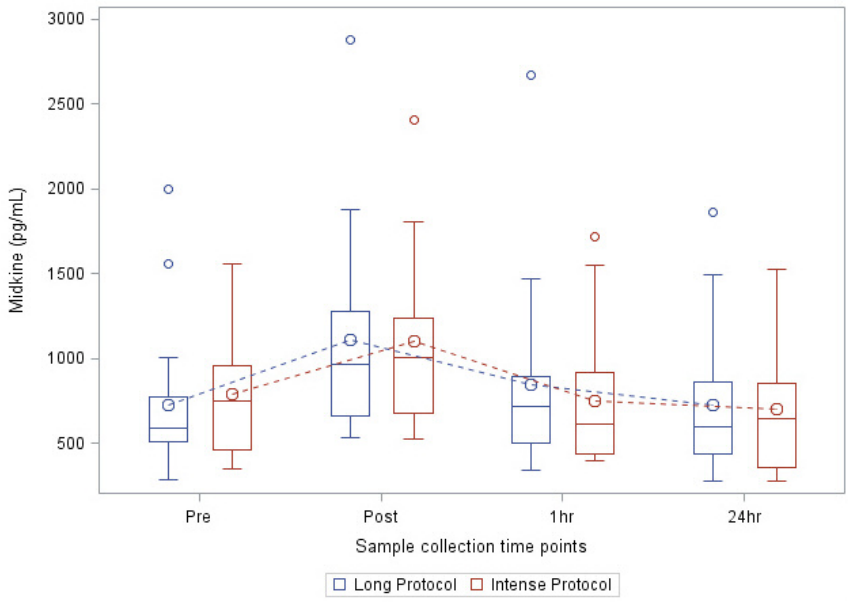

Figure 3: Distribution (median and quartiles in box-plots, mean in circles) of midkine (pg/ml) at each time point for the Long (Blue) and Intense (Red) exercise protocols.

Table 8. Distribution of cytokine polymorphisms in the cohort of athletes (n=16). The minor allele homozygote is identified by grey shading. The NCBI reference number, the polymorphism substitution and the impact of the minor allele on cytokine production are provided. The cytokines have been classified as having pro- (PRO), anti-inflammatory (ANTI) and regulatory (REG) functions.

Cytokine	Poly-morphism (ancestral > replacement minor allele)	SNP reference number (rs number NCBI)	Impact on cytokine production of allele substitution	Number &% of SNPs in the whole cohort (high/moderate/low expression) (NCBI minor allele homozygote)		
PRO				**high**	**moderate**	**low**
IL-6	G>C	1800795	decreased expression	**GG** 6 38%	**GC** 5 31%	**CC** 5 31%
IL-8	A>T	4073	increased expression	**TT** 5 31%	**AT** 7 44%	**AA** 4 25%
IL-17a	G>A	1974226	?increased expression	**AA** 1 6%	**AG** 4 25%	**GG** 11 69%
IL-17a	A>G	2275913	decreased expression	**AA** **1** **6%**	**AG** **8** 50%	**GG** **7** 44%
ANTI						
IL-10	A>G	1800896	decreased expression	**GG** 0 0%	**AG** 4 25%	**AA** 12 75%
IL-1ra	T>C	419598	decreased expression	**TT** 8 50%	**TC** 7 44%	**CC** 1 6%
REG						
IL-4	C>T	2243250	increased expression	**TT** 0 0%	**CT** 5 31%	**CC** 11 69%
IFN-γ	T>A	2430561	decreased expression	**TT** 2 13%	**TA** 10 63%	**AA** 4 25%

Note: in this study the minor allele for IFN-γ was classified as T and would increase the production of IFN-γ.

individual consultations, and managing teams to prevent and treat common respiratory illnesses that can impair training and competitive performance. This study was undertaken to develop a combined clinical and laboratory-based paradigm to identify athletes at higher risk of respiratory illness associated with respiratory infections and/or inflammation. The three key elements of the predictive model of increased risk of upper respiratory tract illness were low levels of IgA in pre exercise saliva samples, detection of viral EBV DNA in saliva, and a genetic predisposition for pro-inflammatory cytokine responses.

There was a higher prevalence of low levels of salivary IgA in the illness-prone athletes but the group median concentrations were not significantly different between the groups. This outcome highlights the need for monitoring of changes in salivary IgA in individual athletes rather than cohort means or medians. Although salivary IgA is a promising biomarker (58) it exhibits substantial within- and between-subject variability (13). There was also a trend for higher detection rates of EBV-DNA in saliva of the illness-prone group. This is consistent with previous studies of high detection rates of EBV-DNA prior to the appearance of URS in illness-prone athletes with low levels of salivary IgA (28).

There was insufficient power to examine the potential risk of the cytokine response to the standardised exercise protocols (data not shown) as the technology used proved inadequate for the detection of many of the low levels of plasma cytokines. However, a predisposition to pro-inflammatory responses has been identified in previous studies of long-distance runners (11) and these responses need to be re-examined in other sporting disciplines. The resting and post-exercise levels of C-reactive protein did not correlate with infection risk and supports previous findings (10) that C-reactive protein is not a good biomarker for identifying athletes at risk of URS.

The cytokine polymorphisms indicated a possible association with higher prevalence of the minor alleles for IL-6 and IFN-γ in the illness-prone athletes and an increasing risk score with the total number of minor cytokine alleles overall. These patterns support previous studies (5) and indicate athletes with a genetic predisposition to pro-inflammatory cytokines responses or impaired anti-inflammatory response (5, 61) may have a higher risk of URS.

The response of midkine to the two exercise protocols showed a rapid substantial increase

Table 9. Number (and percentage) of minor alleles in the healthy and illness-prone athletes for each cytokine, mean and the composite genetic risk score for minor alleles and difference between groups (mean (SD)). Comparisons between groups with significant differences or trends are indicated *.

Cytokine	SNP ID	Number of minor alleles (Risk Score)	Healthy (n=12)	Illness prone (n=4)	Total population	p-value
IL-4	rs2246250	0	8 73%	3 27%	11 69%	0.76
		1	4 80%	1 20%	5 31%	
		Genetic risk	0.3 (0.5)	0.3 (0.5)	0.3 (0.5)	0.77
IL-6	rs1800795	0	3 50%	3 50%	6 38%	0.15
		1	4 80%	1 20%	5 31%	
		2	5 100%		5 31%	
		Genetic risk	1.2 (0.8)	0.3 (0.5)	0.9 (0.9)	**0.06***
IL-8	rs4073	0	4 80%	1 20%	5 31%	0.40
		1	6 86%	1 14%	7 44%	
		2	2 50%	2 50%	4 25%	
		Genetic risk	0.8 (0.7)	1.3 (1.0)	0.9 (0.8)	0.37
IL-10	rs1800896	0	8 67%	4 33%	12 75%	0.18
		1	4 100%		4 25%	
		Genetic risk	0.3 (0.5)	0.0 (0.0)	0.3 (0.4)	0.21
IL-17a	rs2275913	0	5 71%	2 29%	7 44%	0.83
		1	6 75%	2 25%	8 50%	
		2	1 100%		1 6.3%	
		Genetic risk	0.7 (0.7)	0.5 (0.6)	0.6 (0.6)	0.66
IL-17a	rs1974226	0	7 64%	4 36%	11 69%	0.30
		1	4 100%		4 25%	
		2	1 100%		1 6.3%	
		Genetic risk	0.5 (0.7)	0.0 (0.0)	0.4 (0.6)	0.17
IL-1ra	rs419598	0	5 63%	3 38%	8 50%	0.49
		1	6 86%	1 14%	7 44%	
		2	1 (100%		1 6.3%	
		Genetic risk	0.7 (0.7)	0.3 (0.5)	0.6 (0.6)	0.27
IFN-γ	rs2430561	0	1 25%	3 75%	4 25%	**0.03***
		1	9 90%	1 10%	10 63%	
		2	2 100%		2 13%	
		Genetic risk	1.1 (0.5)	0.3 (0.5)	0.9 (0.6)	**0.01***
Minor alleles	**Genetic risk score**	Mean (SD)	5.6 (2.1)	2.8 (1.75)	4.9 (2.3)	**0.03***

immediately after exercise that returned to baseline concentrations within 24 h post-exercise. The changes in midkine concentrations most likely reflect responses to the renin-angiotensin pathways that control midkine activity (42). There were no significant differences in the responses of the illness-prone and healthy athletes, and therefore the midkine responses do not provide additional information to a paradigm for risk of URS. The marked elevation in midkine concentration after exercise probably reflects short-term mobilisation of the protein sequestered in tissue such as endothelial cells lining blood vessels rather than endogenous synthesis of midkine (42).

Table 10: Increased risk of URS for each individual SNP minor alleles and the composite genetic risk score for the cumulative addition of minor alleles. Univariate Odds Ratios (OR) and 95% confidence intervals (CI) derived from exact logistic regression.

Cytokine	SNP ID (NCBI)	OR (95% CI) for 3 or more URS episodes	p-value
IL-4	rs2246250	0.68 (0.01, 12.4)	1.00
IL-6	rs1800795	0.18 (0.00, 1.34)	0.13
IL-8	rs4073	2.06 (0.36, 16.7)	0.58
IL-10	rs1800896	0.46 (0.00, 3.37)	0.54
IL-17a	rs2275913	0.64 (0.04, 6.11)	1.00
IL-17a	rs1974226	0.37 (0.00, 1.96)	0.36
IL-1ra	rs419598	0.27 (0.00, 3.12)	0.51
IFN-ɣ	rs2430561	0.05 (0.00, 0.96)	0.05
Minor allele score	**Genetic risk score**	0.49 (0.15, 0.98)	0.04

The clinical assessments were unremarkable, and the only relevant finding was a tendency towards a higher prevalence of a combined history of asthma and allergy to inhaled allergens in the athletes with a lower incidence of URS, although the numbers were small. The records of current medications indicated that these athletes were well managed therapeutically for medical conditions suggesting the lower URS incidence may be the consequence of good clinical management. Knowing the EBV serology status was important for interpreting the EBV-DNA data but otherwise there were no pathology tests of significance to the risk paradigm. The history of URS in the 12 months prior to the study did not correlate with the prospective data recorded during the study and confirmed by the sports physician, suggesting a self-reported history of URTI may not be a good indicator of potential future risk of URS.

We have taken the outcomes of this study, combined them with previous published data, to formulate a framework for categorising underlying factors associated with recurrent URS as uncontrollable, controllable, or partially controllable risks (Table 12). This table provides a checklist for clinicians and trainers working with highly trained athletes, and researchers investigating the causes, diagnosis, treatment and management strategies for common respiratory illnesses experienced by athletes.

Uncontrollable risks

The uncontrollable risks include genetic risks associated with an individual's predisposition to a pro-inflammatory response. Inherited cytokine polymorphisms will influence the cytokine response to infections and other inflammatory stimuli. Characterising the underlying genetic risk may be beneficial for identifying an athlete at risk of pro-inflammatory responses. Despite the small cohort size in this study, the genetic risk scores from the cumulative addition of minor alleles for each cytokine SNP provided some indication of an increased risk of URS with a higher number of minor alleles. Significantly larger cohort studies are required to confirm this outcome. However the outcomes are consistent with previous studies examining individual cytokine gene polymorphisms (5), suggesting the high-expression genotype for IL-6 may be associated with an increased likelihood of >3 URS/year. The study also indicated a possible association with the IFN-ɣ genotype which is associated with increased severity of illness symptoms (57). A defect in IFN-ɣ secretion has also been identified in athletes presenting with persistent fatigue and impaired performance (3).

As the cytokine responses to exercise are determined by the genotype these could also be classified as uncontrollable risks. In this investigation there was insufficient data and power to reliably assess the cytokine responses by the cyclists to the two exercise protocols. A previous study of long-distance runners identified impaired inflammatory regulation in illness-prone athletes (11), with higher levels of IL-6 and lower levels of IL-10 and IL-1ra after intense exercise. Further studies are required to determine if these cytokine responses to a standardised exercise test can be included in a risk assessment paradigm. Genetic differences in IL-10 SNPs

Table 11. Midkine concentration (pg/mL) pre-exercise and the post-exercise change for all athletes undertaking the Intense and Long Exercise Protocols at each collection time (Figure 3).

Time point	Intense Protocol			Long Protocol		
	Median (95% CI)	Change (95% CI)	*p value*	Median (95% CI)	Change (95% CI)	*p value*
Pre-exercise	750 (460, 958)	257 (+220, +400)	<0.0001	590 (+513, +865)	359 (+247, +505)	<0.0001
Post-exercise	1007 (681, 1237)	-289 (-414, -188)	0.0001	969 (+665, +1294)	-272 (-318, -174)	<0.0001
1 h Post	614 (435, 917)	-342 (-480, -212)	0.0002	716 (+563, +912)	-345 (-483, -177)	<0.001
24 h Post	648 (358, 851)	-89 (-162, +188)	0.32	600 (+475, +999)	-11 (-112, +117)	0.89

Table 12. Controllable and Uncontrollable risk factors for upper respiratory symptoms in athletes.

Risk and Category	Description of Risk for URS	References
Uncontrollable Risks		
Genetic risk	High expression IL-6 genotype	Cox *et al* (2010) (5) Current study
	Low-expression IL-2 genotype	Cox *et al* (2010) (5)
	High expression IL-10 genotype	Zehsaz *et al* (2014) (61)
	Low expression for IFN-ɣ (minor alleles)	Current study
	Genetic risk score for cumulative number of minor cytokine alleles	Current study
	Gender	He *et al* (2014) (30)
Immune responses to exercise	Low resting IL-8, IL-10, IL-1ra levels	Cox *et al* (2007) (11)
	Low post-exercise IL-10, IL-1ra	Cox *et al* (2007) (11)
	High post-exercise IL-6	Cox *et al* (2007) (11)
	Post-exercise IL-10 production	Gleeson *et al* (2011) (21)
Controllable Risks		
Health Risks	Asthma	Reid *et al* (2004) (49) Cox *et al* (2008) (9)
	Allergy to inhaled allergens	Schwellnus *et al* (1997) (51) Katelaris *et al* (2000) (34) Katelaris *et al* (2003) (35) Cox *et al* (2010) (7)
	Immunodeficiency and autoimmune states	Fricker *et al* (1999) (16) Reid *et al* (2004) (49)
	Vaccinations	Gärtner and Meyer (2014) (18)
	Hygiene	Pyne *et al* (2000) (47)
	Nutritional status	Gleeson & Pyne (2015) (27)
	Low Vitamin D	He *et al* (2013) (31)
	EBV status and viral reactivation	Gleeson *et al* (2002) (28) Cox *et al* (2004) (8)
	Psychological stress	Konig *et al* (2000) (38)
	Sleep disturbance	Konig *et al* (2000) (38)
Partially Controllable Risks		
Environmental Risks	Winter season	Konig *et al* (2000) (38) Hellard *et al* (2015) (32) Svendsen *et al* (2016) (55)
	Cold air exposure	Passali *et al* (2004) (45)
	Extreme temperatures (hot/cold)	Walsh *et al* (2011) (58)
	Exposure to infections	Pyne *et al* (2000) (47)
	International air travel	Svendsen *et al* (2016) (55)
Training and Competition	Respiratory epithelial damage due to airway drying or trauma	Passali *et al* (2004) (45) Martin *et al* (2012) (40)
	Circadian rhythm disturbance	Reilly *et al* (2007) (50)
	Low salivary IgA levels and excretion rates	Gleeson *et al* (1999) (26) Walsh *et al* (2011) (58) Neville *et al* (2008) (43) Ihalainen *et al* (2016) (33)
	Low tear fluid SIgA levels	Hanstock *et al* (2016) (29)
	Training monotony	Svendsen *et al* (2016) (55)

have been associated with susceptibility to URS due to impaired IL-10 responses (5, 61), making IL-10 a candidate cytokine gene for further investigation.

Controllable risks

A comprehensive clinical assessment can assist with identifying and managing clinical conditions associated with URS. Ensuring appropriate therapeutic control of asthma and allergy to inhaled allergens can reduce the risk of upper respiratory illness and associated fatigue (1, 35, 49). The suggestion of a higher incidence of allergy in the athletes with a lower incidence of illness in this study may indicate these conditions were well controlled in these athletes, as the majority had rhino-conjunctivitis and recorded use of relevant therapeutics. Other controllable underlying health risks include ensuring adequate levels of Vitamin D (31) and management of psychological stress (38).

Determining the EBV serology status assists with managing athletes. Those athletes who are seronegative can be advised and managed to avoid primary infection. Seropositive athletes can be monitored for viral reactivation, such as expression of EBV-DNA in saliva. This study identified a higher expression rate of EBV-DNA in the illness-prone athletes. It is unlikely that a therapeutic intervention would be implemented but the EBV can be controlled with anti-viral therapy (8). Detection of EBV-DNA is not routinely available but this study confirmed previous reports of lower salivary IgA levels in illness-prone athletes prior to the detection of EBV viral reactivation (28) and the availability of point-of-care salivary IgA tests (4) make this a useful biomarker. However, as highlighted in this study, EBV seronegative subjects can still be at risk of recurrent URS from other causes (Table 2). Even with the small number of subjects in this study, there was a trend for an association of low levels of salivary IgA with an increase in illness (Table 5). Monitoring tear fluid (29) or salivary IgA (4, 23, 33, 43) can assist in modifying training regimes to limit the impact of upper respiratory symptoms associated with immune activation/inflammation on training and competitive performance.

Partially controllable risks

While the season cannot be controlled, knowing there is an increased risk for URS associated with training and competing during the winter months (32, 38) can assist

with modifying training and implementing personal avoidance strategies for at-risk athletes. The impact of cold air on mucosal membranes has not been extensively studied but is known to increase the symptoms that mimic respiratory infections (36). Similarly, risks of some infections can be reduced by prior vaccination (18), reducing exposure to potential pathogens and implementing personal hygiene strategies (47).

The Challenges

The challenges facing researchers involved in studies of high-performance athletes include the selection of appropriate performance and laboratory tests, limitations of some analytical methods, self-reported clinical data, sample size estimation and recruitment of sufficient subjects, and statistical analyses in small sample sizes to provide meaningful outcomes. This study experienced all these challenges. The ability to recruit an adequate sample size that had the statistical power proved a challenge for this complex study design, particularly given the extended study period. In this study the requirement for daily reporting over a 9 month period impacted negatively on the recruitment process. In turn, this limitation impacted on the statistical power of the study outcomes. Based on the most commonly measured parameter and assuming a mean difference in salivary IgA concentration of ~10-15 mg/L and a standard deviation of ~10 mg/L in a parallel groups design, a minimum of 15 subjects would be needed in both healthy and illness-prone groups.

This study was costly, not only for the exercise and laboratory testing components, but labour intensive, as it involved exercise physiologists, sports physicians and multiple experienced laboratory scientists, as well as web designers for the 9 month prospective on-line reporting, and statisticians. To overcome one of the criticisms of investigations of self-reported upper respiratory illness by elite athletes, this study included physician verification of the symptoms for each reported episode. This added to the commitment by the athletes and the sports physicians but was deemed important for collection of higher order clinical data. Additional pathology testing, such as determination of IgE-specific antibodies for aero-allergens, infectious serology and pathogen identification would have been informative but expensive.

An unexpected challenge was the selection of the analyser for the simultaneous measurement of the multiple cytokines. The analyser proved unsuitable for the assessment of the plasma cytokine responses to the exercise protocols, with most cytokine levels being below the limit of detection of the assay, highlighting the need to ensure any new technology is appropriate for the study sampling regime. The lack of cytokine responses limited the interpretation of the cytokine genotypes and relationships to URS in this cohort.

CONCLUSIONS

Paradigms for assessing the risk of respiratory illness in high-performance athletes historically have not been well defined. High performance athletes experiencing recurrent respiratory illness should be assessed clinically and monitored to eliminate or reduce controllable risks. The laboratory parameters identified with an increased risk of illnesses included cytokine gene polymorphisms for the high expression of IL-6 and IFN-ɣ, expression of EBV-DNA in saliva, and low levels of salivary IgA. SNP analysis is not routinely performed and monitoring viral reactivation is usually confined to research settings. Recent development of point-of-care analysers for salivary IgA allows for real-time assessment of the risk of URS in individual athletes and may prove beneficial for adoption of preventive strategies.

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Exercise induced alterations in NK-cell cytotoxicity - methodological issues and future perspectives

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Abstract

With their ability to recognize and eliminate virus-infected and neoplastic cells, natural killer cells (NK-cells) represent an important part of the innate immune system. NK-cells have attracted the attention of exercise scientists for more than thirty years ago. To date, it is widely accepted that NK-cell counts in the peripheral blood are strongly influenced by acute exercise. Additionally, many studies reported effects of both, acute and chronic exercise on NK-cell cytotoxicity. However, these findings are contradictory. The inconsistence in findings may be argued with different exercise paradigms (type, duration, intensity). Moreover, strongly varying methods were used to detect NK-cell cytotoxicity. This review gives an overview of studies, investigating the impact of acute and chronic exercise on NK-cell cytotoxicity in young and old healthy adults, as well as on specific populations, such as cancer patients. Furthermore, different methodological approaches to assess NK-cell cytotoxicity are critically discussed to state on inconsistent study results and to give perspectives for further research in this field.

Key words: exercise, physical activity, NK-cell, NK-cell cytotoxicity, NKCA

Introduction

Natural killer cells (NK-cells) are part of the innate cellular immune system and have the ability to recognize and eliminate tumor- and virus-infected cells as well as parasites and some types of bacteria.

NK-cells belong to the lymphocytes and its phenotype ($CD56^+$, $CD3^-$) is defined by expression of CD56 and lack of CD3 which is a T-cell surface marker. There are two subpopulations of NK-cells. The first subset is referred to as $CD56^{bright}$ NK-cells due to their high-density surface expression of CD56. They display a low cytotoxic capacity and a high secretion rate of cytokines in response to activation. $CD56^{bright}$ NK-cells represent the minority of the NK-cells and occur mainly in secondary lymphoid tissues (SLT). $CD56^{dim}$ NK-cells represent the majority of the NK-cells in blood (about 90%), spleen, and bone marrow. The amount of CD56 is lower on their surface. However, they characterized by a high cytotoxic capacity (10, 14, 70).

After recognizing and binding to the target cells, NK-cells release a diversity of cytokines, such as interferon-gamma (IFN-γ), tumor growth factor-beta (TGF-β) and interleukin-10 (IL-10) (13, 14, 16). Additionally, they secrete cytotoxic agents such as perforin and granzyme B which are released from cytolytic granules by directed exocytosis (28). IFN-γ increases the activity of other NK cells and activates the innate and adaptive immune system by the stimulation of macrophages and enhancing the cytotoxicity of $CD8^+$ T-lymphocytes(61). TGF-β and IL-10 are immune regulators and have the ability to suppress the immune system.

The activation of NK-cell effector function is regulated by the balance of activating (e.g. CD16, KIR2DS, NCRs, NKG2D, NKp30) and inactivating (e.g. certain KIR receptors, KLRG1, NKR-P1, NKG2A) signals of cell surface receptors, recognizing structures of high molecular weight (38).

NK-cells have attracted the attention of exercise scientists more than 30 years ago. Several studies have shown that absolute and relative NK-cell counts in peripheral blood are strongly influenced by acute physical exercise. Increased NK-cell numbers immediately after cessation of exercise have commonly been reported (69). Depending on the exercise regime (type, duration, intensity), a decrease of NK-cell numbers has been described after a delay of at least 15-30 minutes. This decrease can persist more than 24 hours.

More recent studies have revealed that NK-cell subsets differentially respond to exercise stimuli. Evidence suggests that NK-cells are mobilized from the spleen into circulation by epinephrine dependent β-adrenergic signaling (42). As reported by Dimitrov and colleagues, this mobilization primarily affects cytotoxic ($CD56^{dim}$) NK-cells and is driven by a specific expression of the cell surface markers CD11a and CX3CR1 (15). The knowledge about the redistribution of NK-cells after a delay of exercise is still sparse. Exercise-induced muscle-derived IL-6 was proposed to promote NK-cell infiltration in tumor tissue (53).

Since increased physical activity levels improve survival rates in several neoplastic diseases (51) and elevated NK-cell

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numbers in tumor tissue are associated with a better prognosis (18, 19, 58), NK-cells became one promising target to explain the positive effect of exercise on cancer patients` survival. Furthermore, it was hypothesized that an exercise-induced enrichment of NK-cells could be used for an isolation of these cells in view of further immunotherapeutic strategies (e. g. transplantation) (3).

Besides the intermediate exercise-induced alterations in NK-cell counts, many studies have reported acute and chronic functional changes of NK-cells in response to exercise. However, the results of these studies are inconsistent. In this review a distinction was made between acute effects (single bouts of exercise) and chronic effects (interventions) of exercise on NK-cell cytotoxicity (NKCA) in different populations (young healthy adults, older healthy adults, patient populations). Furthermore, the results of these studies will be discussed against the background of different methodological approaches for detecting NKCA and their translational/clinical relevance.

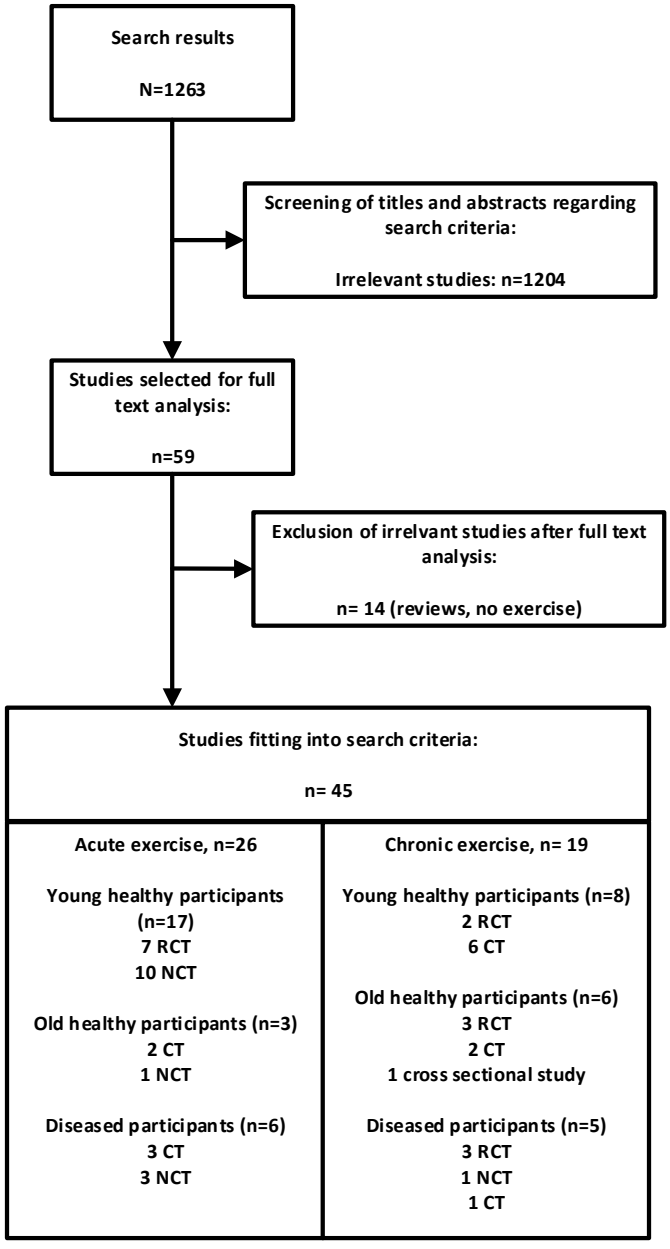

Figure 1: Literature search and results

Methods

A literature search was performed in Pubmed in April 2016. Titles and abstracts were retrieved and screened by three independent reviewers (MK, AS, PZ). The search strategy consisted of a combination of database-specific MeSH terms, free text, and Boolean operators (“AND”, “OR”, “NOT”). The detailed search strategy was performed with the following words: exercise, physical activity, sport, training, natural killer cells, NK-cells, cytotoxicity, cytotoxic, natural killer cell cytotoxic activity, cytolytic activity, NKCA, NK cell function.

Studies were defined as being either randomized controlled trial (RCT), controlled trial (CT), non-controlled trial (NCT), or cross-sectional (observational) study.

Acute exercise was defined as a single bout of exercise followed by assessment of immunological parameters. Chronic exercise was repeatedly performed in the context of an exercise intervention program. Studies on both, acute and chronic exercise employed different types of participants that have been divided into three groups (young and healthy; old and healthy; not healthy). The programs are shortly described by duration, intensity and type of exercise. Moreover, the methods of NKCA determination, the measurements time-points, the outcomes as well as the results are summarized in table 1 and 2.

Results

An overview of literature search and results is given in figure 1.

Acute exercise

Regarding acute exercise 26 studies including 502 participants were selected for analysis. Subjects participated in seven RCTs (157 participants), fourteen NCTs (196 participants) and five CTs (149 participants).

Gannon et al. (20) showed an increased NKCA after moderate cycling at 65% VO_2peak that returned to baseline levels two hours after cessation of cycling. Similar results were reported by other studies using endurance exercise intensities of 50-90% VO_2peak and durations of 20-120 minutes (8, 36, 37, 48, 50, 63, 64). Strasner et al. (64) demonstrated an increasing NKCA in high intensity aerobic exercise (80% VO_2max) compared to moderate exercise (40% VO_2max). These results are in line with those of Nieman et al. (48) who reported a more pronounced increase of NKCA after intensive endurance exercise (80% VO_2max) compared to moderate endurance exercise (50% VO_2max).

Similar to many other studies (37, 40, 43, 50, 52), Shek et al. (63)determined an increasing number of NK-cells immediately after cessation of exercise. While all studies mentioned before used aerobic exercise, Lee et al. (29) applicated Qi-training and found no exercise-induced changes in NK-cell counts. Strasner et al. (64) investigated different intensities and revealed an increase of NK-cell counts after high intensity endurance exercise but not after moderate endurance exercise.

Nieman et al. (48) adjusted the NKCA on a per cell level (NKCApC) and showed a significantly increased NKCApC two hours after recovery of high intensive treadmill running. In contrast Lee et al. (29) showed an increased NKCApC

immediately after Qi-training which returned to the baseline after two hours. Despite these contrary results, other studies found no impact of exercise on NKCApC (20, 37, 50, 64).

Comparing NKCA in old and younger subjects, the results of Woods et al. (75) did not indicate any difference in response to exercise. Ogawa et al. (50) also measured no differences in NKCA and NKCApC but a higher increase of NK-cell counts in elderly untrained subjects after exercise.

Few studies investigated the influence of exercise on patients with specific diseases. Yamanka et al. (77) indicated difference between patients with cervical spinal cord injury (CSCI) and healthy subjects. The patients with CSCI had a constant NKCA during the study in contrast to the able-bodied persons with an increased NKCA immediately after exercise on an arm-crank ergometer. In contrast, Ueta et al. (66) mentioned a decreasing NKCA in patients with spinal cord injury and no difference in NK-cell counts. Furthermore, Furusawa et al. (19) demonstrated a decreasing NKCA after a wheelchair marathon.

Ullum et al. (67) compared HIV+ patients with healthy controls and identified an impaired mobilization of NK-cells and less lysis of target cells in HIV+ patients after exercise. Boas et al. (8) compared NK-cell counts and NKCA in patients with cystic fibrosis and healthy control subjects. After exercise to exhaustion on a bicycle ergometer, NK-cell counts increased in both groups but were significantly higher in the healthy control group. Similar results were reported for NKCA.

Chronic exercise

Regarding chronic exercise 19 studies including 781 participants were selected for analysis. Eight studies were characterized as RCTs (335 participants), nine studies as CTs (380 participants), one as cross sectional study (42 participants) and one as NCT (24 participants).

Moro-Garcia et al. (39) showed increased NK-cell counts and a higher NKCA in athletes compared to non-athletes. In line with these results, Pedersen et al. (51) described higher NKCA in trained subjects compared to sedentary controls. Moreover, Nieman et al. (44) reported elevated NKCA in marathon runners in comparison to sedentary controls, but no differences in NK cell counts. Nieman and colleagues reproduced these results in another study comprising of a 15 week supervised walking program (49). Suzui et al. (65) showed an increase of $CD56^{bright}$ NK-cells during as well as at the end of one month of volleyball training with a decreased NKCA during training. Nevertheless, Roberts et al. (58) found no changes in NK-cell counts, as well as NKCA and NKCApC.

Oppositional to studies including healthy young subjects, most studies with healthy elderly participants did not indicate an influence of exercise on NKCA (11, 46, 55, 56). Nieman et al. (46) revealed an increased NKCA in women with a good physical constitution compared to sedentary controls. However, NKCA of sedentary women did not increase after a twelve week training program. Woods et al. (74) and McFarlin et al. (33) showed an increase of NKCA after a six month aerobic exercise program and a ten week resistance training, respectively. Rincon et al. (57) investigated frail elderly participants and reported an increase in NKCApC after a three month exercise intervention.

Fairy et al. (18) and Peters et al. (54) performed a 15 week and seven month cycling program with breast cancer survivors and found an increase of NKCA after the intervention. Unlike these results Nieman et al. (45) found no influence on NK-cell counts and NKCA after an eight week exercise intervention with moderate weight training and aerobic exercise in a comparable population. Na et al. (41)described an increased NKCA in stomach cancer patients which exercised until 14 days post-surgery. Hagstrom et al. (22) conducted 16 week resistance training with breast cancer patients. The authors did not report any changes of CD107a, a marker for degranulation on NK-cells.

Discussion

Impact of exercise on NKCA

In contrast to the commonly reported blood kinetics of NK-cell counts in response to acute exercise, including an increase immediately after cessation as well as a decrease for up to 48 hours, depending on type, duration and intensity of the exercise session (69), data on NKCA are inconsistent. A tendency could be stated in favor of increased NKCA after more intense aerobic exercise (48, 64). However, such conclusions are restricted by a number of methodological limitations which are discussed in the "methodological issue" section. A potential explanation for the reported increased NKCA immediately after cessation of more intense aerobic exercise could be argued by the epinephrine driven increase in circulating $CD56^{dim}$ (15). Indeed, epinephrine levels have been described to increase with aerobic exercise intensity and persist until 15 minutes after cessation (26). In contrast, epinephrine has also been reported to decrease NKCA in vivo, ex vivo as well as in vitro (35, 59). Additionally, epinephrine is known to primarily mobilize NK-cell subsets with a low expression of the activating receptor NKG2D (3). However, our own research suggests that NKG2D expression increased after prolonged aerobic exercise (79).

Besides epinephrine, other stress related factors such as cortisol and prostaglandins (PGE_2) (12), which are also increased during and after aerobic exercise (27, 31, 62), are associated with a reduced NKCA (35). Against this backdrop, the complex kinetics of catecholamines, prostaglandins and glucocorticoids which differ during and after various exercise modalities should be considered in further studies investigating the influence of acute exercise on NKCA. Finally, it is worth mentioning that NK-cells which are collected from blood during or after acute exercise do not necessarily display the NK-cell proportion which is mobilized and especially migrated in the tissue to eliminate neoplastic or virus infected cells. Although speculative, it might be possible that NKCA of NK-cells which have migrated from the blood stream in different tissues in the following 24 hours after cessation of exercise are influenced by several other (local) factors and are completely independent from the known as "stress hormones". In view of the physical fitness level, studies unanimously revealed elevated NKCA in subjects with a good physical constitution (39, 44, 46, 51, 58). Although Nieman and colleagues (44) showed that physical fitness does not affect NK-cell counts, NKCA might also be influenced by the distribution of NK-cell subsets. More precisely, if physical fit subjects would indicate higher proportions of $CD56^{dim}$ NK-cells, this would result in an increased NKCA(65).

Table 1. Studies on acute exercise and NKCA

Authors	*Year*	*Paper title*	*Subjects*	*n*	*Study design*	*Classification*	*Exercise*	*Period, duration, intensity*	*Methods of NKCA measurement*	*Parameters*	*Time of sampling*	*Results*
Acute exercise –Young healthy participants												
Bigley et al.	2014	Acute exercise preferentially redeploys NK-cells with a highly-differentiated phenotype and augments cytotoxicity against lymphoma and multiple myeloma target	healthy, trained, 30y	16	NCT	no	cycling	3x 30min trials with -5%, +5%, +15% of lactate threshold	PBMC. targets: U266; RPMI-8226; 721.221; 221 AEH; K562. Flow cytometry. NK count / NKCA / NKCA per cell	Cytotoxicity in %	Pre, every 10min, Post, 1h	Highly-differentiated (KIR+/NKG2A) NK cells more redeployed. Shift in proportion of NK. Impact on NKCA against HLA-expressing targets. Post NKCApC↓, 1h NKCApC↑. no effect on K562
Gannon et al.	1998	Beta-endorphin and natural killer cell cytolytic activity during prolonged exercise. is there a connection	male, 26y, recrea-tional active	10	RCT	4x10: Placebo exercise trial / Naltrexone exercise trial, control, non exercise trial	moderate cycling	2h, 65% VO_2peak	PBMC. K562. NK count / NKCA / NKCApC	Cytotoxicity in %	Pre, 1, 2, 4, 24h	NKCA ↑ at t1, t2. NKCA ↓ at t4. NKCApC unaltered
Kappel et al.	1991	Evidence that the effect of physical exercise on NK cell activation is mediated by epinephrine	8 untrained healthy men (20-29y)	2x8 (exercise and epinephrine infusion)	NCT	no	cycling	60 min, 75% VO_2max	BMNC. K562. ^{51}Cr release assay. NKCA	Cytotoxicity in %	Pre, Post, 2h	NKCA ↑ significantly during epinephrine infusion as well as during bicycle exercise / after 2h NKCA ↓ below basal in both / at indentical times no significant differences between NKCA with exercise and epinephrine / Study presents that NKCA induced by physical exercise can be mimicked by the infusion of epinephrine
Kakanis et al.	2010	The open window of susceptibility to infection after acute exercise in healthy young male elite athletes	elite male cyclists	10	NCT	10	cycling	2h at 90% second ventilatory threshold	PBMC. K562. Annexin V. Flow cytometry. NK count / NKCA	Cytotoxicity in %, phenotypes ($CD56^{dim}$ and $CD56^{bright}$)	Pre, Post, 2,4,6,8,24h	NK count ↓ from Pre to 4h&8h. After 24h count = baseline. No signif NKCA and $CD56^{dim}$ change. $CD56^{bright}$ count ↑ only immediately post exercise
Lee et al.	2005	Acute effect of qi-training on natural killer cell subsets and cytotoxic activity	healthy men, 26y	18	RCT	9 + 9 control	Qi-training	1h training	PBMC. LDH release of K562. NK count / NKCApC	Cytotoxicity in %	Pre, Post, 2h after	NKCApC ↑ 60% and returned to baseline within 2h. NK cell count unchanged
McFarlin et al.	2003	Repeated endurance exercise affects leukocyte number but not NK cell activity	young men	10	NCT	4x 10	cycling	3x 20min including 2x 4h recovery	Whole blood. K562 ^{51}Cr release assay. NKCA	Cytotoxicity in %	Pre, Post, 2h, 24h. Pre2, Post2, 2h2, 24h2	NKCA ↑ Post. Returned to baseline after 2h. Greater elevation upon afternoon exercises than upon morning exercises
Miles et al.	2002	The relationship of natural killer cell counts, perforin mRNA and CD2 expression to post-exercise natural killer cell activity in humans	18-40y, moderately trained male runners	10	RCT	6 + 4 control	running	60min,tread mill at 80% VO_2peak	Whole blood. K562 ^{51}Cr release assay. NK count / NKCA / NKCApC	Cytotoxicity in %	Pre, Post, 1,5h, 5h, 24h	NKCApC unchanged. NKCA ↑ by 63% Post; ↓ by 42% at 1,5h in RUN group due to numeric redistribution
Millard et al.	2013	Brief Exercise Increases Peripheral Blood NK Cell Counts without Immediate Functional Changes, but Impairs their Responses to ex vivo Stimulation	25-40y	29	NCT	no	running 50-80 sec	run up+down 150 stair-steps	NK cell isolation: MACS. K562. ^{51}Cr release assay. NK count / NKCApC	Cytotoxicity in %	Pre, Post	NK number ↑. NKCApC not altered

Authors	Year	Paper title	Subjects	n	Study design	Classification	Exercise	Period, duration, intensity	Methods of NKCA measurement	Parameters	Time of sampling	Results
Moyna et al.	1996	Exercise-induced alterations in natural killer cell number and function	healthy male and female 1:1	64	RCT	exercise group, control group	cycling	18min: 3x 6min at 55, 70, 85% VO_2peak	Whole blood ^{51}Cr release assay. K562. NK count / NKCA	Cytotoxicity in %	Pre, 6min, 12min, 18min, 2h	Alterations of NK number (x10) not accompanied by changes of a similar magnitude in NKCA (2x). NKCA ↑. After 2h at baseline
Nieman et al.	1993	Effects of high- vs moderate-intensity exercise on natural killer cell activity	trained men, 17-31y	10	RCT	2x10	moderate treadmill 50% VO_2max vs high intensity 80%	45min	PBMC. K562. ^{51}Cr release assay. NK count / NKCA / NKCApC	Cytotoxicity in %, lytic units	Pre, Post, 1, 2, 3,5h	Moderate: NKCA ↑ Post, below baseline at 1h & 2h. No change of NKCApC. Intense: NKCA ↑ Post, below baseline at 1h & 2h. Significant ↑ of NKCApC from Post to after 2h recovery
Nieman et al.	1995	The acute immune response to exhaustive resistance exercise	male, 47y. 9y weight training	10	NCT	no	parallel leg squat	10 rep at 65% 1-RM every 6sec. 3min rest, new set. until muscular failure. ->9700 +/- 1570kg, 98 +/- 14 rep. 45% VO_2peak	PBMC. K562. ^{51}Cr release assay. NK count / NKCA / NKCApC	Cytotoxicity in %, lytic units	Pre, Post, 2h	NKCA 61% ↓ from Pre to 2h Post. NKCApC ↓ ~40% below Pre level for at least 2h. Suggest prostaglandine from neutrophils and monocytes suppress NKCA. Leg squat exercise to muscular failure results in response of circulating immune cells, like high intense endurance exercise, despite lower % VO_2max and hormonal response
Nieman et al.	2006	Immune changes: 2h of continous vs. intermittent cycling	male trained cyclists, 21y	12	NCT	2x12: continuous cycling vs intermittent cycling	cycling	2 h at 60-65 % $Watt_{max}$. Continuously or with 3min of Rest period every 10 minutes (total time 2h 33 min) 75% VO_2max.	PBMC. K562 labeled with DiO and PI. Flow cytometry. NK count / NKCA	epinephrine, cortisol, interleukins	30 min before exercise, Post and 1h after	No diff in pattern of change between C and R exercise trials. NKCA ↑ Pre to Post. ↓ from Post to 1h below baseline
Pedersen et al.	1988	Modulation of natural killer cell activity in peripheral blood by physical exercise	Healthy, male (23-26y)	6	NCT	no	a) cycling b) back-muscle training	a) 60 min 80% VO_2max b) 5 sets Intervall 10 min = 300 contractions in 1h	BMNC. K562. ^{51}Cr release assay. NK count / NKCA	Cytotoxicity in %	Pre, Post, 2h, 24h	a) NKCA ↑ Pre to Post. Below basal level after 2h. Returned to baseline after 24h b) no significant influence
Shek et al.	1995	Strenuous exercise and immunological changes: a multiple-time-point analysis of leukocyte subsets, CD4-CD8 ratio, immunoglobulin production and NK cell response	male, 22y	6	NCT	no	cycling	2h at 65% VO_2max	PBMC. K562. ^{51}Cr release assay. NK count / NKCA	Cytotoxicity in %	Pre, 30,60,90,120, 150,180,210,240min, 1d, 7d	NK number and NKCA ↑ during exercise. Persistent depression in post-exercise period. 40% ↓ of NK count and NKCA for as long as 7 days. Overtraining -> immunosuppression?

Authors	Year	Paper title	Subjects	n	Study design	Classification	Exercise	Period, duration, intensity	Methods of NKCA measurement	Parameters	Time of sampling	Results
Strasner et al.	1997	Effects of exercise intensity on natural killer cell activity in women	women, 21-33y, oral contraceptives	8	RCT	3x 8 high vs moderate vs control	cycling 80% VO_2max, 40% VO_2max, control	25min per session	PBMC. K562. ^{51}Cr release assay. NK count / NKCA	Cytotoxicity in %	Pre, Post, 90min, 3h	High-Int: NK number ↑and NKCA ↑ post exercise, NKCApC slightly ↑. NKCA ↓ at 90min, NK number like baseline. no diff at 3h. Moderate Intensity: no diff. from control at any time
Wang et al.	2008	Exercise affects platelet-impeded antitumor cytotoxicity of natural killer cell	sedentary men, 22y	37	RCT	ME= moderate exercise SE= servere exercise WUE-SE= severe exercise after warm-up exercise	cycling	ME: 60% VO_2max for 40min SE: up to VO_2max 40min WUE-SE: up to VO_2max + warm-up	target: nasopharyngeal carcinoma cells. Isolation of NK cells (MACS). Flow cytometry. NK count / NKCA	perforin, granzyme B, NK-NPC-binding, caspase activation	Pre, Post	Severe exercise NK count ↑and enhanced NKCA (perforin, granzyme B content) and promotes the platelet-impeded apoptosis induced by NK. Warm-up reduces resistance of platelets increasing NKCA after severe exercise
Wang et al.	2009	Systemic hypoxia affects exercise-mediated antitumor cytotoxicity of natural killer cells	sedentary men, 22y	16	NCT	6x16	cycling	HighE 21%O_2, Mod.E 21%, ME15%, ME12%, breathing in15% and 12% O_2.	nasopharyngeal carcinoma cells. Flow cytometry. NK isolated by MACS. NK count / NKCA	NK-NPC-binding, cellular perforin and granzyme B	Pre, Post, 2h	HE 21%: perforin/granzyme B/IFN in NK, capacity of NK to bind to NPC↑. Breathing at12/15% O_2: no influence. ME 12/15% O_2: NK count, perforin/granzyme B/IFN-g, NK-NPC binding↑
Acute exercise - Old healthy participants												
Bigley et al.	2015	The effect of age and latent cytomegalovirus infection on nk-cell phenotype and exercise responsiveness in man	young ~30y, older ~56y	40	CT	12 CMV+ young, 12 CMV- young, 8 CMV+ old, 8 CMV- old	cycling	30min, 80% VO_2max	PBMC. Flow cytometry. NK count	CD57, CD158, $CD56^{dim/bright}$, KLRG1	Pre, Post, 1h	CMV blunts NK redeployment in young and old. Relatively less $CD57^+$ and $CD158^+$. CMV^{neg} old subjects showed largest NK mobilization. CMV-independent ↑ of $CD57^+$ NK cells during aging. Data suggests: CMV ↓ NK surveillance after exercise in young and old
Ogawa et al.	2005	A single bout of exercise influences natural killer cells in elderly women, especially those who are habitually active	women: trained by walking 64y; untrained 63y; young untrained 25y	24	NCT	8, 8, 8	treadmill walking	a single 30min exercise, 70-75% VO_2peak	BMNC. ^{51}Cr release assay. K562. NK count / NKCA / NKCApC	Cytotoxicity in %	Pre, Post, 2h	↑ of NK count of untrained elderly was higher post-exercise than those of other groups. No difference in NKCA and NKCApC among the three groups. Suggest defect in cytotoxic ability in sedentary elderly; Natural immunity enhanced in daily exercising elderly

Authors	Year	Paper title	Subjects	n	Study design	Classification	Exercise	Period, duration, intensity	Methods of NKCA measurement	Parameters	Time of sampling	Results
Woods et al.	1998	Effects of maximal exercise on natural killer (NK) cell cytotoxicity and responsiveness to interferon-alpha in the young and old	young (18-27y) and old (58-77y), sedentary	47	CT	14 young and 33 old	treadmill, running	2,5 (old) /4mph speed, with 2% increase every 2min until exhaustion.	^{51}Cr release assay. K562 and Daudi cells. PBMC. NK count / NKCA / NKCApC	Cytotoxicity in %	Pre, Post	No diff in NKCA against K562 or Daudi between old and young despite signif higher % of NK cells in old. Maximal exercise -> NKCA↑; Correlation NKCA / NK number in the young, not in the old. Maximal exercise NKCApC ↑against Daudi, not against K562. (IFN to augment NKCA is impaired in the old)
Acute Exercise – Diseased participants												
Bigley et al.	2015	Acute exercise preferentially redeploys NK-cells with a highly-differentiated phenotype and augments cytotoxicity ... Part 2. Impact of latent cytomegalovirus infection and catecholamine sensitivity	(healthy), trained, 30y, with CMV infection	(16) + 6 neue	NCT	no	cycling	LT test; 3x 30min trials: -5%, +5%, +15% LT	PBMC. targets: U266; RPMI-8226; 721.221; 221 AEH; K562. Flow cytometry. NKCA / NKCApC	Cytotoxicity in %	Pre, every 10min, Post, 1h	CMV impairs NK mobilization with exercise when intensity exceeds LT. Latent CMV abated post increase in NKCA. CMV compromises NK cells after acute exercise. Impaired β-AR signaling?
Boas et al.	1999	Immune modulation following aerobic exercise in children with cystic fibrosis	15 subjects with cystic fibrosis and 15 healthy controls	30	CT	patients with CF and healthy controls	cycle ergometer (60 r.p.m.)	max.exercise test to the exhaustion. Power of the ergometer was increased every minute for 10, 15 or 20 W based on the stature of the subjects	PBMC. K562 labeled with PHK-2; PI. Flow cytometry. NK count / NKCA	Cytotoxicity in %	Pre, Post, 60min	Cellular immune response to acute exercise in children with mild or moderate CS appears broadly normal
Furusawa et al.	1998	Short-term attenuation of natural killer cell cytotoxic activity in wheelchair marathoners with paraplegia	spinal cord injuries, wheelchair male marathoners, 27-52y	16	CT	9 + 7 controls with SCI	Wheelchair marathon race		PBMC. ^{51}Cr release assay. T cell leukemia cell line MT2. NK count / NKCA	Cytotoxicity in %	Day before, Post and 1 day after the race	Number of NK cells and NKCA significantly ↓ after the race and returned to Pre-level after 24 h. ↑ post-race adrenaline level, but NKCA ↓. ↓ of NK/NKCA due to overtraining, not due to SCI. Cortisol level ↑ post
Ueta et al.	2008	Attenuation of natural killer cell activity during 2-h exercise in individuals with spinal cord injuries	Subjects with spinal cord injuries (SCI)	13	NCT	7 SCI + 6 able-bodied control	arm ergometer	2h at 60% VO_2max	PBMC. ^{51}Cr release assay, T cell leukemia cell line MT2. NK count / NKCA	Cytotoxicity in %	Pre, 60min, Post	Able-bodied: NKCA ↑ at 60min of exerc, Post and 2h after end of exerc. PGE2 unchanged. SCI: NKCA higher than control at baseline. NKCA ↓ Post, recovered at 2h after exerc. NK cell number lower than in able-bodied and unchanged throughout the experiment. PGE2 ↑ Post, returned to baseline 2h after exerc. Suggested that ↑ of PGE2 in SCI partially contributes to NKCA reduction.

Authors	*Year*	*Paper title*	*Subjects*	*n*	*Study design*	*Classification*	*Exercise*	*Period, duration, intensity*	*Methods of NKCA measurement*	*Parameters*	*Time of sampling*	*Results*
Ullum et al.	1994	The effect of acute exercise on lymphocyte subsets, natural killer cells, proliferative responses, and cytokines in HIV-seropositive persons	8 HIV postiv (26-38yr)	16	CT	8 HIV+ and 8 controls	cycling	60 min, 75% VO_2max	BMNC. ^{51}Cr release assay. K562. NK count / NKCApC	Cytotoxicity in %	Pre, Post, 2h, 4h	Suggestion: HIV+ subjects have impaired mobilization of neutrophils, NK and LAK cells
Yamanaka et al.	2010	Impaired immune response to voluntary arm-crank ergometer exercise in patients with cervical spinal cord injury	persons with CSCI (cervical spinal cord injury) land dysfuncional sympathetic NS, male, chronic injury state	14	NCT	8 patients with CSCI + six able-bodied persons	arm crank ergometer	20 min of exercise with 60 % of VO_2max	PBMC. ^{51}Cr release assay. T cell leukemia cell line MT2. NK count / NKCA	Cytotoxicity in %	Pre, Post, 1h, 2h	Able-Bodied: - NK cell count and NKCA ↑ post-exercise and ↓ 1h later to a lower level than before returning to the baseline after 2 h Conclusion: In subjects with CSCI, lack of NKCA response is probably due to dysfunctional sympathetic NS: no adrenaline response

Table 2. Studies on chronic exercise and NKCA

Authors	*Year*	*Paper title*	*Subjects*	*n*	*Study design*	*Classification*	*Exercise*	*Period, duration, intensity*	*Methods of NKCA measurement*	*Parameters*	*Time of sampling*	*Results*
Chronic Exercise – Young healthy participants												
Moro-Garcia et al.	2014	Frequent participation in high volume exercise throughout life is associated with a more differentiated adaptive immune response	athlets and non-athlets	95	CT	30 young non-ath.; 27 young ath.; 26 elderly non-ath.; 12 elderly ath;	young ath.: rowing, running, resistance training; Old ath.: easy-moderate intesity	young ath: 6d/wk; 2h/d; Old ath: 5d/wk; 80min/d	Whole blood. Flow cytometry. CD107a expression for degranulation. CD69 "NK activation". NK count / NKCA / NKCApC	Cytotoxicity in %	Pre, Post	NKCA ↑; young athletes: NKCA and degranulation significantly increased; young ath. had higher NK counts than old ath; no change in count or structure of NK receptors
Nieman et al.	1990	The effects of moderate exercise training on natural killer cells and acute upper respiratory tract infection	mildy obese women, 25-45y	36	RCT	18 exercise + 18 control	walking	15 weeks; 45min/d brisk walking at 60% max HR; 5d/wk	PBMC. K562. ^{51}Cr release assay. NK count / NKCA	Cytotoxicity in %	Pre, week 6 and 15	NKCA rose strongly after 15 weeks to the same level, in control and exercise group! Fewer upper respiratory tract infection symptoms in exercise group.
Nieman et al.	1995	Immune function in marathon runners versus sedentary controls	marathon runners, ~40y	40	CT	22 + 18 sedentary controls	Conditions: training >4y,>7marathons in less than 3h45min	-	PBMC. Flowcytometry. K562.^{51}Cr release assay. NK count / NKCA / NKCApC	Cytotoxicity in %	one sample	NKCA/NKCApC significantly different: in marathoners elevated. NK cell number similar; suggest chronical elevation; %body fat and VO_2max related antiproportional to NKCA
Pedersen et al.	1989	Natural killer cell activity in peripheral blood of highly trained and untrained persons	male racing cyclists (median 23y), healthy control (median 26y)	42	CT	27 trained + 15 untrained	performance test	none	PBMC. K562.^{51}Cr release assay. NK count / NKCA	Cytotoxicity in %	one sample	NKCA higher in trained persons
Roberts et al.	2004	CD94 expression and natural killer cell activity after acute exercise	highly trained male triathletes, 20-30y	9	RCT	no	Training for competition. test cycling: 20min submaximal exercise, then incremental test until exhaustion	10 weeks; 3x test cycling in lab	PBMC. Flow cytometry. K562.^{51}Cr release assay. NK count / NKCA / NKCApC	Cytotoxicity in %	week 2,5,10: Pre-exercise; 20min after exhaustion	Resting NK numbers and NKCA did not differ over 10 weeks; NK numbers increased post-exercise; Increased NKCA after exercise reflects numbers of NK cells. NKCApC not changed
Suzui et al.	2004	Natural killer cell lytic activity and CD56(dim) and CD56(bright) cell distributions during and after intensive training	female college-level volleyball players + healthy students as control	15	CT	8 + 7 control	heavy pre-season training	1 month: 5h/d; 6d/wk.	PBMC. Flow cytometry. K562.nonradioactive Europium release assay. NK count / NKCA / NKCApC	Cytotoxicity in %	Pre, day 10, one day before end; 1wk after end of training	NKCA ↓ from Pre to End, returned to Pre-level 1wk later. Similar for NKCApC. $CD56^{bright}$NK↑, $CD56^{dim}$ NK number unchanged

Authors	*Year*	*Paper title*	*Subjects*	*n*	*Study design*	*Classification*	*Exercise*	*Period, duration, intensity*	*Methods of NKCA measurement*	*Parameters*	*Time of sampling*	*Results*
Wang et al.	2011	Hypoxic exercise training promotes antitumour cytotoxicity of natural killer cells in young men	sedentary men	60	CT	5 groups with 12	21% O_2 control; 15% O_2 control; 21% O_2 50% max work rate; 15% O_2 50% heart rate; 15%O_2 50% max work rate;	30min/d, 5/wk, 4 weeks.	nasopharyngeal carcinoma cells (NPC). PBMC. NK isolation with MACS-negative immunomagnetic selection. NK count / NKCA	Perforin & granzyme B with flow cytometry; annexin V,propidium iodide staining (FACS) -> % necrotic/apoptotic cells.	48h before and 48h after last training	15% O_2exercices reduce terminally differentiated NK subsets; activating molecules and cytotoxic granule proteins in NK ↑, but no increased anti-NPC-cytotoxicity of NK; $CD56^{dim}$ increased, $CD56^{bright}$ decreased; increase of CD11a and NKG2D; anti-NPC cytoxicity increased
Chronic Exercise - Old healthy participants												
Campbell et al.	2008	Effect of exercise on in vitro immune function: a 12-month randomized, controlled trial among postmenopausal women	postmenop overweight /obese, 50-75y women, healthy, sedentary	115	RCT	53 + 62 control	Moderate aerobic /bicycling exerc. (Beginning: 40% max HR; 60-75% in week 8); C: strechting, relaxing	1 year; Exerc: >45min/d, 5d/wk; C: 1d/wk	PBMC.Flow cytometry. K562 - propidium iodide assay. NK count / NKCA / NKCApC	Cytotoxicity in %	Pre, 3 mo, 12 months.	no effects
McFarlin et al.	2005	Chronic resistance exercise training improves natural killer cell activity in older women	65-86y postmenop women	25	CT	19 + 6 control	resistance training	10 weeks; 3x/wk; 80% 1.RM (first repetition maximum)	Whole blood. K562.^{51}Cr release assay. NK count / NKCA / NKCApC	Cytotoxicity in %	before: Pre, Post, 2h; after 10 wk: Pre, Post, 2h	No significant difference in NKCA but NKCA ↑ in response to an acute bout of exercise
Nieman et al.	1993	Physical activity and immune function in elderly women	sedentery women; 67-85y	30 + 12 + 13	CT	14 walkers (sedentary), 16 control; + 12 highly conditioned; + 13 young healthy not active	walking	30-40min 5d/wk. 12 weeks. 60% heartrate	PBMC. Flow cytometry. K562.^{51}Cr release assay. NK count / NKCA / NKCApC	Cytotoxicity in %	Pre, 5wk, 12wk; (old active at baseline; young inactive at 12wk)	Walkers: no improvement in NK activity after 12 weeks. Highly conditioned at baseline: higher lytic units than walkers despite no diff in NK numbers. Seasonal effects on immune functions
Raso et al.	2007	Effect of resistance training on immunological parameters of healthy elderly women	sedentary women; 60-77y	42	RCT	exercise + control	moderate resistance training	12 mo; 3 sets of 12 repet at 60% 1.-RM for 5 diff exercises; 3x/wk; 60 min/d;	PBMC. Flow cytometry. K562; ^{51}Cr release assay. NK count / NKCA / NKCApC	Cytotoxicity in %	Pre, 6mo, 12mo	No significant difference between groups or according to time for quantitative ($CD56^{dim/bright}$, CD3, ..) and functional immunological (NKCA, ..) parameters
Raso et al.	2012	Immunological parameters in elderly women: correlations with aerobic power, muscle strength and mood state	sedentary elderly women, 60-77y	42	Cross-sectional	-	none	none	PBMC. Flow cytometry. K562.^{51}Cr release assay. NK count / NKCA / NKCApC	Cytotoxicity in %, muscle strength, aerobic power, mood state	one sample	Neither NKCA nor lymphocyte proliferation were correlated with aerobic power or muscle strength; Psychological changes associated with aging may have a substantial adverse effect upon the immune system, and immunological function may be enhanced more by addressing these issues than by focusing upon aerobic or resistance training

Authors	Year	Paper title	Subjects	n	Study design	Classification	Exercise	Period, duration, intensity	Methods of NKCA measurement	Parameters	Time of sampling	Results
Woods et al.	1999	Effects of 6 months of moderate aerobic exercise training on immune function in the elderly	sedentary elderly 65y	29	RCT	14 + 15 control	moderate aerobic exercise	6 months: 3x/wk; at 50% to 65% VO_2max; 10-40min/d;	PBMC. K562. Flow cytometry.^{51}Cr release assay. NK count / NKCA / NKCApC	Cytotoxicity in %	Pre-exercise, post, 20min after exercise	No significant difference in NKCA. Acute exerc response is attenuated in Control and exercise groups post-intervention. NK function was performed only on 7 + 12 subjects
Chronic Exercise – Diseased participants												
Fairey et al.	2005	Randomized controlled trial of exercise and blood immune function in postmenopausal breast cancer survivors	postmenop 50-69y, breast cancer survivor	53	RCT	25 cyclists + 28 control	cycling; 70-75% VO_2max	15wk; 3x/wk; wk1-3: 15min; incremental, wk13-15: 35min;	PBMC. K562.^{51}Cr release assay. NK count / NKCA / NKCApC	Cytotoxicity in %	Pre, week 15.	NKCA ↑
Hagstrom et al.	2016	The effect of resistance training on markers of immune function and inflammation in previously sedentary women recovering from breast cancer: a randomized controlled trial	breast cancer survivor; 18-70y; sedentary	39	RCT	20 + 19 control	resistance training	16 wk; 60min 3x/wk; repetions at 80% 1-RM;	Flow cytometry	markers of NKCA, granzyme B, perforin	Pre; week 17	No change in NK-percentage. No change in granzyme B or perforin. reduced NK cell expression of TNF-α
Na et al.	2000	Exercise therapy effect on natural killer cell cytotoxic activity in stomach cancer patients after curative surgery	stomach cancer patients, 28-75y	35	CT	17 exersice + 18 control	arm + bicycle ergometer	from post-OP day 2: 30 min 2x/d, 5d/wk for 2 weeks. 60% maxHR	PBMC. K562.^{51}Cr release assay. NKCA	Cytotoxicity in %	Post OP days 1, 7, 14	Suggests early moderate exercise has benefincial effect on NK in Sto.Cancer patients after surgery. NKCA in younger and non-metastasis patients more increased
Nieman et al.	1995	Moderate exercise training and natural killer cell cytotoxic activity in breast cancer patients	female breast cancer; undergone surgery, chemo, and/or radiation previously; 35-72y	12	RCT	6 +6 control	moderate weight training and aerobic activity	8 wk; 60min/d; 3d/wk ; 75% HR max;	PBMC.Flow cytometry. K562.^{51}Cr release. NK count / NKCA / NKCApC	Cytotoxicity in %	Pre, Post	NKCA and NK number not significantly altered; Suggests: moderate exercise over 8weeks no significant effects
Peters et al.	1994	Influence of a moderate exercise training on natural killer cytotoxicity and personality traits in cancer patients	breast cancer patients; 49 +/- 6y; stage one or two. >6 months since surgery	24	NCT	no	moderate cycling	7 months; 2-3x week;	NK isolated according to Cosentino and Cathcart. K 562.^{51}Cr release assay. NK count / NKCA / NKCApC	Cytotoxicity in %	Pre; 5 weeks; 7 months;	After 7 months: NKCA of patients in range of healthy people from other studies
Rincon et al.	1996	Exercise in frail elderly men decreases natural killer cell activity	frail male, >70y	13	CT	6 + 7 control	strength, balance, walking, stretching	3months; 60min/d; 3x/wk	Whole blood. Flow cytometry. K562.^{51}Cr release assay. NK count / NKCA / NKCApC	Cytotoxicity in %, lytic units	each Pre + Post: 0wk, 6wk, 12 wk	Exercise increased NKCA transiently Pre/Post; But long-term effect: reduction below basal NKCA; Caution in very frail elderly

In view of chronic exercise interventions, results are contradictory. The heterogeneity in results could also be argued by alterations in "stress hormones". Chronic alteration in baseline levels and differences in the response to acute exercise after training periods in catecholamine-, prostaglandin- and glucocorticoid levels have been reported in several studies with healthy subjects (9, 32, 80). For example, regular exercise is known to reduce resting glucocorticoid- and catecholamine levels. Therefore, decreased levels of these agents which can be found in subjects with a good physical constitution or after a specific exercise intervention could explain an improved NKCA although the proportion of $CD56^{bright}$ may increase (65). When investigating clinical populations, it should be kept in mind that baseline levels and responses to exercise of the named factors are further influenced by several diseases (17, 80). As already mentioned for acute effects of exercise, these factors should be investigated as mediators of alterations in fitness/training-induced NKCA as well.

Methodological issues

Regarding functional NK-cell assessments several approaches have been described. The NK cell function was commonly tested by measuring the NK-cell cytotoxic activity (NKCA), NK cell count and NKCA per cell. Cytotoxic activity assays were frequently performed by mixing either peripheral blood mononuclear cells (PBMC) or isolated NK cells with a target cell line (leukemia cell line K562 in most cases). The percentage of target cell lysis was frequently measured with ^{51}Cr release assay detecting the radioactivity in the samples supernatant (47, 50, 67). However, newer studies utilized non-radioactive agent like Annexin V which was determined by flow cytometry (25). Some research groups had concerns about K562 as a HLA-deficient target cell line. Therefore they used further cell lines with different surface expression patterns like Daudi cells, MT2, U266, RPMI-8226, 721.221, and 221 AEH (4, 75, 77).

Other studies assess the NKCA without counting killed target cells, but by measuring the amount of perforin, granzyme B, IFN- γ, and the NK-target-cell-binding via flow cytometry (73). Further indirect measurements can complete the evaluation of NK cells. The differentiation marker CD57 can be used as target for flow cytometry. CD57 expression is induced on $CD56^{dim}$ NK cells after activation by IL-2. CD57+ $CD56^{dim}$NK-cells are considered to be terminally differentiated and mature. They are characterized by poor cytokine-mediated proliferation, a higher sensitivity to stimulation via CD16 and higher cytotoxicity (30). Moreover, the lysosomal-associated membrane protein-1 (LAMP-1 or CD107a) was reported as marker of NK-cell cytolytic activity. Its surface expression was increased by engaging MHC devoid targets and its expression levels correlated with both, cytokine secretion and lysis of target cells. However, a large NK-cell subset did express CD107a while it did not secrete cytokines. Therefore, it was suggested that CD107a could be used as marker of NK-cell activity and identification of a large degranulation fraction of activated NK-cells (1).

As pointed out in table 1 and table 2 several different approaches have been used to assess NKCA. Against this background, results of studies are hardly comparable. NKCA was frequently measured using PBMCs (4–7, 11, 18–20, 29, 41, 43–49, 51, 55, 56, 58, 63–66, 72, 74, 75, 77) whereas other studies incubate tumor cells with whole blood samples (33, 34, 36, 39, 40, 57, 57). These approaches have some major limitations. First, both methods include other cells than NK-cells with tumor-competitive properties, such as cytotoxic T-cells. Therefore, statements on specific functional changes of NKCA are restricted. Second, the use of whole blood samples comprises various other agents, such as cytokines and hormones which may influence the target cells itself. However, Gotlieb and colleagues suggest that in vitro and ex vivo assays usually lead to an overestimation regarding the reported "stress hormone" induced suppression of NKCA (21). The authors propose that further research should use whole blood sample approaches, arguing that such attempts reflect the in vivo situation more precisely. We absolutely agree with this opinion. Nevertheless, one should keep in mind that incubating tumor cells with whole blood samples does not represent the in vivo situation (tumormicromilieu) as well.

Third, it is worth to mention that NKCA should be quantified on a per cell level. This is relevant since NK-cell numbers can strongly vary between pre- and post-exercise conditions. Just in a few studies NK-cells were isolated (e. g. by magnetic beads) to detect cytotoxicity (25, 37, 54, 71, 73). To minimize NK-cell-specific alterations, a negative selection is strongly recommended.

Furthermore, studies showed that NK-cell subset distribution is influenced by both, acute and chronic exercise (25, 65, 72). Since NK-cell subsets display different cytotoxic potentials, changes in these fractions should also be considered when analyzing NKCA.

Another issue, which might be of clinical relevance, is the type of tumor cells which is used as target for detecting NKCA. Especially in clinical studies with cancer patients, e. g. breast cancer, it would make sense to measure NKCA against a breast cancer cell line, whereas using the leukemia cell line K562 is of inferior interest (with the exception of the genesis of secondary neoplastic burdens). This issue becomes even more important since first studies have shown that NKCA depends on the type of target cells (e. g. nasopharyngeal carcinoma cells (71–73), Daudi (75), U266 (4, 5), RPMI-8226 (4, 5), 721.221 (4, 5), 221AEH (4, 5) or T-cell leukemia cell line MT2 (19, 66, 77)).

Some studies determined NKCA by measuring the amount of perforin, granzyme B, IFN-γ and the NK-target-cell-binding via flow cytometry (73). To get more knowledge about the mechanistic underpinnings, a combination of both direct and indirect methods seems to be a promising strategy for further research. In addition, the expression of activating and inhibiting NK-cell-receptors should be taken into account. Exercise has been described to alter NK-cell receptor expression (79). Therefore, changes in NK-cell target killing might not be reasoned by in- or decreased levels of cytotoxic agents, but by a modification of surface receptor expression.

Finally, acute effects of NKCA can persist longer than 24 hours (63). Therefore, measurement time points in studies investigating chronic effects of exercise should be chosen carefully (measurements up to 24 hours after the last training session might still display acute effects).

Due to heterogeneous methods, strongly varying exercise interventions and measurement time points, we decided that quantitative analysis (meta-analysis) does not make sense so far.

Although the significance of current literature on the influence of exercise on NKCA is restricted and needs further approval, there is evidence that at least some positive effects, such as an improved defense against neoplastic cells is based on NK-cell mobilization and activation (53). In fact, increased NK-cell numbers in tumor tissue are associated with improved prognosis in different cancer species (23, 24, 68). Moreover, exercise is known to have preventive effects regarding cancer risk and to reduce cancer specific mortality (2, 60, 76, 78). Therefore, research on NK-cells in the context of exercise and cancer was and will be a highly relevant topic for further investigations.

Conclusion

In summary, at least some exercise/training modalities seem to impact NKCA. As potential mediators of these effects, the role of catecholamines, prostaglandins as well as glucocorticoids warrants further investigation. On a molecular level, epigenetic alterations might be involved in functional changes of NK-cells. Currently, exercise studies on NKCA are hard to compare since different exercise regimes (type, duration, intensity, and frequency) were used. Varying measurement time points as well as the use of different methods to assess NKCA delimitate the comparability of the studies. Independently of the methods which will be used to detect NKCA in the future, an additional characterization of NK-cell subsets as well as the assessment of potential mediators (e. g. epinephrine, cortisol) is strongly recommended. Further research is needed to clearly identify the impact of exercise on NKCA.

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Hematopoiesis with Obesity and Exercise: Role of the Bone Marrow Niche

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ABSTRACT

Hematopoietic stem and progenitor cells (HSPC), the most primitive cells of the hematopoietic system responsible for maintaining all mature blood cells, display the hallmark characteristics of self-renewal and multi-potent differentiation into mature cell lineages. HSPC activity is directed by the bone marrow niche, a complex environment composed of heterogeneous cell populations that regulate HSPC function through the secretion of a wide array of cytokines and growth factors. Diet induced obesity results in a dramatic remodeling of the bone marrow niche, skewing HSPC function resulting in a compromised immune system. Exercise is a viable treatment option for deficits imposed by obesity and to combat immune dysfunction; however, the impact of exercise on the bone marrow niche is not well defined. This review summarizes the available information on how obesity disrupts the normal bone marrow niche and HSPC function. In addition, we review the limited data available detailing how exercise may be used to combat obesity induced bone marrow dysfunction, and discuss future directions for research in this field.

Keywords: Exercise training, MSC, HSC, diet-induced obesity

INTRODUCTION

Physical inactivity and a sedentary lifestyle along with caloric over-consumption are major contributors to the global obesity epidemic. The World Health Organization estimates that obesity rates have rapidly increased in recent decades, resulting in 39% of adults being classified as overweight and 13% as obese in 2014 (181). The obesity epidemic is even more prevalent in more developed countries. For example, over 36% of adults in the United States are obese (109). Obesity is associated with an increased risk of health complications including type II diabetes (25, 157), cancer (21, 96, 124), and cardiovascular disease (118, 124). In addition, obese individuals are more susceptible to infection (94, 112, 141, 154) and have worse disease prognosis (147); suggesting impaired immune competence results from obesity. Indeed, obesity is characterized by an increased quantity of innate immune cells (42), and decreased repertoire of functional adaptive immune cells (57) resulting in decreased immune surveillance. Interestingly, these changes to the hematopoietic system mirror the phenotype observed with aging suggesting that obesity may be inducing a premature aging phenotype. The altered production of mature immune cells in obesity merits closer investigation into the precursors of leukocytes: hematopoietic stem and progenitor cells (HSPCs).

HSPCs are the most primitive cells of the hematopoietic system from which all cells in the myeloid, lymphoid, and erythroid lineages are derived. The bone marrow is the primary site of HSPC maintenance and differentiation. Within the bone marrow, the "stem-cell niche" or bone marrow niche is characterized as a specialized microenvironment that maintains HSCs throughout the lifespan (131). The bone marrow niche tightly regulates HSC function through direct cell to cell contact, sympathetic stimulation, and the secretion of autocrine and paracrine factors. Chronic disease states, such as obesity and cardiovascular disease, as well as advancing age, dramatically remodel the bone marrow microenvironment and corresponding milieu resulting in altered HPSC function (34, 35, 110, 136, 169). The increasing number of individuals becoming obese and entering advanced age necessitates further investigations into interventions aimed at attenuating or mitigating detrimental changes to the hematopoietic system.

Exercise bestows numerous benefits that extend the quality and quantity of life in both healthy and diseased individuals. For instance, exercise has been demonstrated to reduce the accumulation of body fat (158), mitigate low-grade chronic inflammation (161), improve cognitive function in healthy (10, 28) and diseased individuals (62, 72), and increase bone density (107). While the complete pleiotropic impacts of physical activity and exercise in healthy and diseased populations are still being determined, it nevertheless remains a low cost, easily implementable, and effective method for attenuating deficits resulting from disease states such as obesity. Although exercise does appear to be a promising therapy, a paucity of data exists examining the impact of exercise on HSPCs and the bone marrow niche. Thus, the purpose of this review will be to unravel how the combination of obesity and exercise impact the hematopoietic system via modulation of the HSPC niche. The following sections will define HSPCs and their regulation by the bone marrow niche, the impact of

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obesity and exercise on HSPCs and the bone marrow niche, and describe possible mechanisms responsible for altered hematopoietic function.

Defining HSPCs

HSPCs are the pluripotent precursors to all mature cells of the hematopoietic system. HSPCs constitute a small fraction, <0.001%, of the total bone marrow cell population, and yet maintain the entirety of the hematopoietic system by undergoing self-renewal divisions to maintain the more primitive populations and differentiation to lineage committed cells (15). HSPC migration, proliferation and fate decisions are regulated by autocrine and paracrine factors from within the bone marrow, as well as systemic cues. Indeed, HSPC mobilization and function is affected by circadian rhythms (91), acute infection (71), psychological stress (125), ischemia (129), tissue damage (114), and energy status (30, 88). The hematopoietic system turns over between 10^{11} – 10^{12} cells daily. To maintain this constant demand throughout the lifespan, a portion of HSPCs remain quiescent for protection against DNA damage or premature exhaustion while more differentiated progenitors maintain mature lineages (69, 100, 146).

Characterization of distinct HSPC populations remains difficult due to a lack of specific known markers. Additionally, difficulty in obtaining HSPC samples from humans has led to most functional research being conducted in murine models. While the murine model is efficient for modeling HSPCs and the bone marrow niche, special considerations must always remain due to differences between species. Indeed, human and murine HSPCs exhibit different cell surface phenotypes. Within mice, the broad HSPC pool is identified by the expression of surface antigens cKit and Sca-1 while lacking the expression of committed lineage markers (LSK) (48). The identification of the SLAM family allowed for phenotypic characterization of HSPC sub-populations that related to reconstitution ability in serial transplant assays (77). Similar HSPC populations have been determined in humans based upon the expression of CD34 (33, 153) and CD38 (152). Traditionally, HSPCs have been divided into different sub-populations of long-term hematopoietic stem cells (LT-HSC,), short-term hematopoietic stem cells (ST-HSC), and multipotent progenitors (MPP) before terminally committing to myeloid and lymphoid lineages (63, 83) (Figure 1). Functionally, the self-renewal capacity is highest among the primitive LT-HSCs while the proliferative capacity is highest among the more differentiated multi-potent progenitors.

Currently, technical challenges make determination of self-renewal and proliferative capacity of HSCs difficult. The

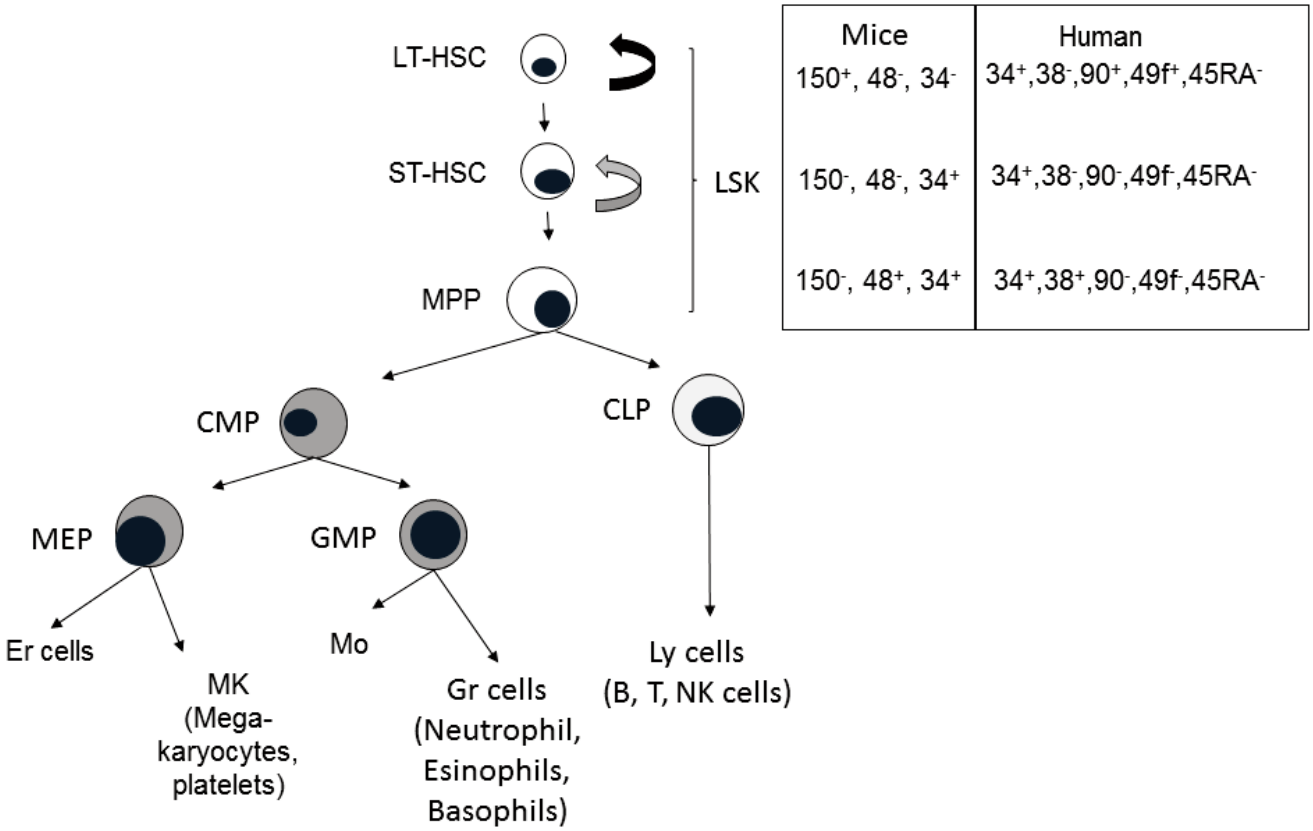

Figure 1. Traditional view of hematopoietic stem and progenitor cell (HSPC) hierarchy. HSPCs have the capacity to self-renewal to maintain primitive cell populations or differentiate into mature hematopoietic cells. The lineage- Sca-1 cKit+ (LSK) phenotypic markers are used to identify the whole stem cell pool within mice. Additional phenotypic markers are outlined that identify distinct HSPC subpopulations in humans and mice. Abbreviations: Long-Term (LT), Short-Term (ST), Hematopoietic Stem Cell (HSC), Multi-Potent Progenitor (MPP), Common Myeloid Progenitor (CMP), MEP (Megakaryocyte/Erythroid Progenitor), GMP (Granulocyte/Macrophage Progenitor), Ertythroid (Er), MegaKaryocyte (MK), Mo (Monocyte), Granulocyte (Gr), Common Lymphoid Progenitor (CLP), Lymphocyte (Ly).

"gold standard" for measuring HSPC functionality relies on the serial transplant assay, whereby bone marrow cells are serially transplanted into lethally irritated mice to determine reconstitution potential (122). This is a time-consuming assay that lasts at least 8 months. Additionally, transplantation into lethally irradiated recipients may not be directly related to HSPC functionality in steady state conditions. Similarly, due to the lack of single, specific markers for tracing HSPC subpopulations, HSPC proliferation and the relative contribution of each subpopulation to steady state hematopoiesis remains challenging. Recent techniques permitting HSPC lineage tracing via "barcoding" (146), and the development of artificial 3-dimensional bone marrow niches for the evaluation of HSPC self-renewal and differentiation *in vitro* over a much shorter timespan (31) will allow for significant advancements in our understanding of HSPC biology.

Obesity, Exercise, and HSPCs

The process of hematopoiesis produces all blood and immune cells and occurs within the bone marrow and, under certain conditions, in peripheral tissue compartments such as the spleen throughout the lifespan (8, 166). Diseases associated with chronic inflammation, including obesity, cancer, heart disease, and other chronic inflammatory conditions, such as the late effects of cancer therapy, have all been linked to alterations in immune function (108, 120, 164). Moderate intensity exercise has been shown to be a powerful mediator of immune function (180). Acute exercise increases the quantity of circulating monocytes and induces an acute inflammatory response that is dependent on exercise intensity (105, 113). Exercise training increase erythrocyte content within peripheral circulation (61), is generally thought to be anti-inflammatory, and enhances tissue regeneration (116, 160). However, the effect of obesity and exercise on hematopoiesis itself remains less well-characterized.

Obesity and HSPCs

Obesity is well documented to increase the quantity of circulating white blood cells in humans (46, 103, 119, 167) and mice (149). Within the bone marrow, diet-induced obesity (DIO) causes an expansion in bone marrow cellularity and mature immune cells (22, 35, 155). This expansion is likely due to the activation and increased cycling of HSPCs. Mice fed a 60% high fat diet (HFD) for 12 weeks experienced an increase in the number of HSPCs and monocyte progenitors (135). This differentiation bias towards myelopoeisis was also observed in a serial transplantation assay (135). Similarly, a shift towards increase myeloid cells and reduction in lymphoid cells was observed in mice after 6 weeks of consuming a 60% HFD (2). On the other hand, mice fed a 45% HFD for 18 weeks experienced a decrease in the most primitive HSPCs and increase in MPPs (14). Additionally, serially transplanted HSPCs displayed a reduced capacity for hematopoietic reconstitution (14). While differences in fat content of the diets used (45% vs 60%) may influence the degree of phenotypic response, the available data suggests DIO stimulates hematopoiesis with a bias towards myelopoiesis, and cycling of HSPCs with exhaustion of the most primitive population. Indeed, DIO appears to stress the HSPC populations risking premature exhaustion and is similar to the phenotype observed in aging.

Acute Exercise and HSPCs

Currently, only a few studies have examined the effects of acute exercise on hematopoiesis. Acute aerobic exercise stimulates HSPCs in the bone marrow as evidenced by an increase in cycling HSPCs and colony forming-unit (CFU) capacity (50, 90, 97). Mooren and Kruger observed an increase in progenitor cells 24 hours following a novel bout of treadmill exercise in sedentary mice while exercised mice experienced no change in HSPCs (97). Together, these data suggest that an acute bout of exercise is a potent physiological stressor, which induces proliferation of the HSPC populations. The effects of acute exercise on hematopoiesis in humans is lacking due to the difficulty of sampling bone marrow tissue. Nevertheless, Wu and colleagues did examined the amount of hematopoietic cells from bone marrow aspirates after an acute bout of exercise in bone marrow donors (171). The authors found that following exercise, while the volume of bone marrow aspirate, number of collections required, and collection pain were improved in the exercise group, no differences were seen in the relative proportion of CD34+ cells (171). However, the total quantity of HSPCs was not evaluated, and more specific markers of HSPCs were not used. Additionally, Wu and colleagues collected bone marrow aspirate 15 minutes after the exercise bout, rather than previous rodent studies that have analyzed bone marrow 24-48 hours after exercise. Together, these data suggest that an acute bout of exercise is sufficient to stimulate proliferation of HSPCs; however, it may take 24-48 hours for changes in HSPC content to be detected.

A larger body of human data exists detailing the mobilization of HSPCs into circulation following acute exercise (39, 49). While the majority of these studies suggest that exercise increases HSPC mobilization in humans, a couple of recent studies have shown that colony forming capacity is decreased in the peripheral blood following acute exercise suggesting decreased function. Kroepfl *et al* (78) showed that HSPCs collected 10 minutes post exercise had a decreased functional capacity, and confirmed these results in a later study investigating the relationship between HSPCs and exercise-induced norepinephrine increase (78). It is possible that during or after acute exercise, more differentiated HSPCs are recruited to peripheral tissues, such as muscle, to participate in tissue repair (114). The mechanisms responsible for acute exercise-induced HSPC mobilization remain to be elucidated. Acute exercise results in an acute inflammatory response that may contribute to the mobilization of HSPCs from the bone marrow. G-CSF and IL-6 have been observed to increase following acute exercise and may influence HSPC mobilization (17, 148, 163, 173). However, no correlations between mobilized HSPC content and cytokines concentrations have been determined following acute exercise (17). This suggests that circulating cytokines may not be the primary signal inducing HSPC mobilization from the bone marrow in response to exercise. Our lab recently observed an increase in G-CSF, SCF, IL-3 and thrombopoeitin from bone marrow stromal cells after an acute exercise bout in mice that may contribute to shuttling HSPCs into peripheral circulation (50). Furthermore, vascular endothelial growth factor-a (VEGF-a) has been observed to be elevated following exercise in circulation (17) and skeletal muscle (50), suggesting that tissue damage or ischemia may play a role in homing HSPCs to peripheral

tissues. Taken together, these data suggest that tissue damage or local factors within the bone marrow are primarily responsible for mobilizing HSPCs following acute exercise.

Exercise Training and HSPCs

Similar to acute exercise, little data exists examining the effects of prolonged exercise training on hematopoiesis. Chronic exercise training increases blood volume, red cell volume, blood hemoglobin content, and immune function. Additionally, some studies have shown that exercise trained individuals have increased amounts of HSPCs at rest in both the bone marrow and peripheral blood (8, 17, 38). Baker and colleagues investigated HSPC content using CFU assays following chronic exercise training and found that increased hematopoiesis was apparent in both the peripheral blood and the bone marrow (8). These data were confirmed and extended by De Lisio and colleagues who demonstrated that progressive treadmill exercise training increase HSPC content collected from the central bone marrow cavity (or "vascular niche") but not the quantity of HSPCs associated with the inner lining of the bones (the "endosteal niche") (38). Importantly, no difference in the repopulating ability of HSPCs was detected between exercise trained and sedentary mice in a bone marrow transplantation assay (38), suggesting that benefits to HSPCs from exercise training may be due to cell-extrinsic factors, possibly due to alterations in the bone marrow niche. In summary, both acute and chronic exercise seem to stimulate hematopoiesis. More research is needed to fully characterize the effects of exercise on hematopoiesis, as well as to delineate the potential mechanisms responsible for these effects. Additionally, future research is necessary to determine if exercise can offset phenotypic skewing within HSPC populations that occurs in chronic diseased states such as obesity and aging.

The Bone Marrow Niche

The concept of a niche, or distinct microenvironment specialized to support the maintenance and differentiation of HSPCs during steady-state or stress hematopoiesis, was first introduced by Schofield in 1978 (131). Since then, important discoveries by a variety of groups have increased our understanding of the cell-types and molecular signals comprising the HSPC niche within the bone marrow. Several excellent reviews have been recently published outlining the cellular and molecular components of the HSPC niche, and the reader is directed to these reviews for a more comprehensive overview of the niche (4, 16, 58). The bone marrow is comprised of multiple, distinct niches that regulate HSPC function and are continuously being redefined with the advent of more precise visualization and identification techniques. Previous data indicated two main stem cell niches within the bone marrow: the endosteal and perivascular niches (77, 137, 172). The endosteal niche was viewed as a site for quiescent HSPCs due to increased homing following transplantation experiments and because osteoblasts were shown to secrete growth factors that regulate HSPC quiescence (5, 142, 172, 178). However, repeated experiments finding low associations of HSPCs contacting osteoblasts and conditional depletion of mature osteoblasts not effecting HSPC quantity have questioned the essential role of the endosteal niche under homoestatic conditions (further reviewed in (98)).

The conditional deletion of stem cell factor (SCF) from endothelial cells which decreased HSPC quantity and the frequent association of HSPCs with blood vessels and sinusoids within the bone marrow shifted the focus to the perivascular niche (77, 142). Furthermore, subsequent reports emphasized perivascular and endothelial cells as the primary site for modulating HSPC quiescence via CXCL12, SCF, and notch ligands (20, 45, 67, 142). Recently, several studies further refined the perivascular niches and observed quiescent HSPC are localized closely to arterioles while more active HSPCs are located near sinusoidal openings (66, 79). The hypoxic nature of the arteriole niche, due to low perfusion (165) and increased vascular wall integrity of arterioles, results in decreased generation of reactive oxygen species (ROS) within HSPCs (66). Furthermore, the hypoxic nature also implicates hypoxic inducible factor-1 (HIF-1) in maintaining HSPC quiescence as increased oxygen content increases stem cell proliferation and mitotic activity (51). Indeed, HIF-1 expression which is upregulated in ischemic tissues partially regulates CXCL12 expression in these tissues, and may be involved in drawing progenitor cells into peripheral tissues (24). Hypoxic culture maintains HSPC reconstitution potential *in vitro* (68), and conditional deletion of HIF-1α within HSPCs results in decreased ability to fully regenerate the hematopoietic system in serial transplant assays (150). The most primitive HSPCs are maintained near the endosteum where HIF-1 expression is the highest (82) whilst its expression is lower the central marrow cavity (77). While this lends credence to the hypothesis that HIF-1 may be leading to increased HSPC retention within the bone marrow, Levesque et al observed an increase in HIF-1α and VEGF-a within bone marrow lysates following G-CSF mediate mobilization of HSPCs (82). Given they used whole bone marrow lysates, it is impossible to determine which specific cell population was responsible for increased HIF-1, and whether HIF-1 directly resulted in HSPC mobilization, or if it worked indirectly via increasing VEGF-a which is a potent mobiliser of HSPCs (82). Overall, these data demonstrate that increased HIF-1 is necessary for long term maintenance of HSPCs *in vivo* and *in vitro*, however, the role in mobilization still needs further delineation. Conversely, HSPCs located near "leaky" sinusoids have an increased production of ROS, are actively cycling, and are readily able to enter peripheral circulation in response to signals from circulation (66). Likewise, HSPCs have been observed to localize near megakayrocytes (19, 179) while erythropoiesis occur in erthroblastic islands (80, 123).

In addition, a variety of stromal cell populations have been identified using genetic labeling approaches that express either leptin receptor (44, 45) Prx1 (55) nestin (64, 92), platelet derived growth factor receptor-α (117) or CXCL12 (111, 143). All of these cell populations are enriched to varying degrees for osteogenic and adipogenic differentiation capacity and contain fibroblast colony forming cells suggesting they consist of mesenchymal stromal cells (MSCs), and that these populations overlap to some yet unknown extent. These MSC populations have all been shown to directly associate with HSPCs and to secrete paracrine factors that regulate HSPC cell fate decisions (16, 58). Thus, through their capacity to signal directly to HSPCs, or to form mature cellular components of the HSPC niche such as osteoblasts and adipocytes

via their differentiation, MSCs are an important, heterogeneous cellular component of the HSPC niche. Taken together the localization of HSPCs impacts their functional status, and disruption of niche homeostasis can disrupt normal hematopoiesis.

Obesity and Exercise Induced Remodeling of the HSPC Niche

The architecture of the bone marrow influences HSPC activity as the bone marrow niche tightly regulates HSPCs in a dynamic balance between quiescence, self-renewal, and differentiation. MSCs, and their progeny, are particularly important modulators of HSPC function. MSCs contribute to bone turnover by differentiating into osteoblasts and remodel the central marrow cavity through adipogenic differentiation in addition to secreting growth factors to support HSPC maintenance (55, 111). Recent evidence suggests a reciprocal relationship between osteogenic and adipogenic differentiation of MSCs. In mice, diet induced obesity (DIO) disrupts the bone marrow compartment, skewing MSC differentiation towards adipogenic lineages resulting in increased marrow adipose tissue (MAT). This reciprocal relationship is also supported by the accumulation of MAT in both obese humans and mice. In humans, increased MAT is negatively correlated with bone density (168), bone mineral density, and bone formation (134). In mice, 8 weeks of 45% HFD induction led to an increase in MAT and decrease in trabecular bone density (35). Similar results were observed in mice fed similar HFD for 6 (2, 139, 140), 12 (47), 18 weeks (176), or 6 months; however, not all studies have observed a concurrent decrease in bone density (47, 139, 140). The differential response of bone mineral density is likely due to different study designs as Styner and colleagues used a 45% HFD for 6 weeks in female mice (139, 140) compared to a 60% HFD for 6 weeks in 8 week old male mice (47). Thus, the relative proportion of fat in the diet may impact observed results in bone marrow remodeling. MAT accumulation negatively impacts the bone marrow compartment as adipocytes will physically occupy red marrow space and MAT potently secretes pro-inflammatory cytokines, such as IL-6, and TNF-α, as well as free-fatty acids, affecting cells throughout the bone marrow including HSPCs (56). Indeed, MAT is a negative regulator of hematopoiesis as the quantity and repopulating capacity of HSPCs collected from areas of high MAT were decreased compared to HSPCs collected from areas of low MAT (104). The skewed MSC differentiation towards the adipogenic lineage, and increased MAT in obese mice and humans mirror changes to the bone marrow compartment observed with aging (101), suggesting that obesity causes a premature aging phenotype in the bone marrow compartment.

The effect of exercise on the bone marrow niche is less well investigated. Forced treadmill the exercise training was demonstrated to remodel the bone marrow by decreasing MAT, and possibly priming MSC towards the osteogenic lineage (8, 159). Mice exercised via wheel running also experience a decrease in bone marrow adiposity even in the presence of a high fat diet (140) or in the presence of PPARγ agonist (139), emphasizing that both forced (8, 36, 159) and voluntary (139, 140) exercise attenuate the accumulation of MAT within the bone marrow. Furthermore, chronic exercise improves the bone architecture via increased mineral density in combination with decreasing MAT (133, 140), supporting the hypothesis that exercise directs MSCs down osteogenic lineages and a more healthy bone marrow environment (104). Interestingly, exercise training donor mice prior to bone marrow transplant (BMT) did not impact homing, engraftment, or reconstitution in recipient mice (38), suggesting that "preconditioning" HSPCs within donors does not have a large impact on the hematopoiesis upon transplantation. However, HSPC transplant into exercise trained recipient mice resulted in increased reconstitution without affecting homing (36). De Lisio and colleagues also demonstrated decreased MAT accumulation, and decreased apoptosis within the bone marrow after recipients were preconditioned with exercise with no enhanced preservation of $CD45^+$ hematopoietic cells suggesting enhanced survival of non-hematopoietic cells in the bone marrow with exercise were contributing to enhanced reconstitution (36). The decreased apoptosis following BMT observed by De Lisio and colleagues, could be due to an increased production of antioxidant enzymes in the marrow in response to exercise (40). These data suggest that the effects of exercise on HSPCs are likely cell non-autonomous versus cell-autonomous.

Potential Mechanisms Regulating Bone Marrow Remodeling in Obesity and Exercise

The effects of obesity and exercise on the bone marrow microenvironment are likely not due to one specific mechanism, but rather a multitude of changes to systemic and bone marrow environments with the respective conditions. Several signaling pathways have been implicated in the reciprocal differentiation of MSCs towards osteogenic or adipogenic lineages that may contribute towards age and chronic disease related deficits to bone density (12, 13, 26, 59, 99, 138, 174). Cytokines secreted by the bone marrow stroma including transforming growth factor β (TGF-β), bone morphogenetic proteins (BMPs), insulin-like growth factor (IGF), and fibroblast growth factors activate transcription factors Runx2 and Osterix, increasing osteogenic differentiation (81, 95, 102, 177). On the other hand, adipogenic differentiation is supported by the transcription factors CCAT/enhancer binding protein alpha (C/EBPα) and $PPAR_{\gamma}$ (32, 75). The following sections will discuss the research available pertaining to how exercise and obesity impact the bone marrow architecture.

Pro-inflammatory cytokines

Obesity is characterized by systemic low grade inflammation, and an increase in the circulating levels of leptin (52), IL-1β (130), Tumor Necrosis Factor α (TNFα) (56, 151), IL-6 (9, 56), and monocyte chemoattractant protein-1 (MCP-1) (73), all of which may have implications on bone marrow homeostasis. TNFα has been previously been demonstrated to inhibit osteoblastic differentiation of MC3T3-E1 pre-osteoblastic and fetal calvaria precursor cells (53). Additionally, TNFα has been observed to activate NF-κB which prevents osteogenic differentiation via the subsequent inhibition of Runx2 and Osterix (74, 86, 87). Furthermore, TNFα-/- mice displayed increased femoral bone density and decreased MAT following an 18 week 60% HFD. Together, these data suggest an integral role for TNFα signaling and pre-osteoblast cell differentiation. IL-1β and MCP-1 have also been demonstrat-

ed to reduce the osteoblastic differentiation potential of MSCs and decrease bone density (144, 145). These data support the potential role of inflammatory cytokines on skewing MSC differentiation from osteogenic towards adipogenic lineages. IL-6 and TNFα have also been implicated to increase osteoclastgenesis, increasing bone breakdown and inhibiting osteoblastgenesis (1, 170, 175). Within the bone marrow, HFD increases the expression of pro-inflammatory cytokines TNFα, IL-1β, and IL-6 in whole mouse bone marrow isolates (14, 56) and isolated rat MSCs (35). Overall, the available data suggests that both systemic and bone marrow specific elevations in inflammatory cytokines results from obesity. Given the role inflammatory cytokines have in directing MSC differentiation, it is likely these cytokines are contributing to the increases in MAT and subsequent bone marrow niche remodeling observed with obesity.

Chronic exercise training is generally considered anti-inflammatory. Exercise training has been shown to decrease the expression of TNFα, IL-1β while increasing the expression of anti-inflammatory cytokine IL-10 in obese rats and mice (18, 54, 85). Additionally, exercise stimulates the release of IL-10 and IL-1Ra, which have been observed to decrease IL-1β and TNFα production (3, 120). Currently, there is limited research available investigating the role of inflammatory cytokines in response to exercise and obesity in the bone marrow. We have previously observed an acute bout exercise alters the secretome of bone marrow stromal cells (50). Other disease models, such as osteoporosis in rats, have observed an increase in bone mineral density following exercise via wheel running (84). Exercised OVX rats also had decreased levels of IL-1β and IL-6 within bone marrow cells compared to sedentary rats (84). These data support the notion that exercise training may mitigate disease associated decreases in osteoblastogensis, bone mineral density, and the increase in pro-inflammatory cytokines. However, further research is necessary to define the relationship between exercise and inflammatory cytokine mediated remolding of the bone marrow architecture, and its role in hematopoiesis. Additionally, studies specifically characterizing changes to the MSC secretome following exercise training in the presence and absence of obesity are needed.

Oxidative Stress and Reactive oxygen species (ROS)

Reactive oxygen species (ROS) are formed during cellular respiration and are mainly produced by the mitochondria. Physiological levels of ROS play a key role in regulating stem cell fate decisions. Obesity has been associated with increased oxidative stress and the generation of ROS (60). MSC are particularly sensitive to the presence of supra-physiological levels of ROS as seen in chronic inflammation and diseased states (further reviewed in (6)). Several studies have indicated that increased presence of ROS inhibits markers of osteogenic differentiation, increases DNA damage, and spurs adipocyte differentiation of MSCs *in vitro* (7, 121, 132). Indeed, the increased presence of ROS has been observed to shift MSC differentiation towards adipogenic lineages in advanced aging (70, 76). In addition to their effects on MSCs which likely have indirect effects on hematopoiesis, ROS have also been shown to directly influence HSPC function. Increasing endogenous ROS production in HSPCs by TNF-α exposure decreased the reconstitution ability in serial transplant assays that was recovered by blocking ROS production (65). Whole body radiation *in vivo* has been shown to have negative long-term effects on primitive HSCs by decreasing their content and colony forming capacity *in vitro* (27). These effects may be due to increased HSPC senescence (93) induced by persistent oxidative stress (162). Together, ROS may negatively impact hematopoiesis indirectly by promoting MSC adipogenic differentiation, or directly by inducing HSC senescence and prolonged oxidative stress.

Few studies exist characterizing the impact of exercise and the generation of ROS within the bone marrow. De Lisio and colleagues observed exercise training reduced DNA damage and apoptosis signaling within the bone marrow of mice exposed to an acute challenge radiation exposure (40). These results suggest that exercise training confers protection within the bone marrow to the exogenous generation of ROS. These results are further supported by increased protection of circulating lymphocytes to irradiation from exercise trained individuals (156) and skeletal muscle of exercise trained mice (37). While it is tempting to speculate that exercise training may stimulate the production of anti-oxidant enzymes within the bone marrow similar to that seen in skeletal muscle (37), no data are available to confirm this hypothesis. However, the reduction of DNA damage within the bone marrow of exercise trained mice following irradiation does support this hypothesis and may contribute to exercise induced MSC biasing towards osteoblast lineages. Further research will be needed to fully characterize this response.

Other Potential Factors

Exercise and obesity may also influence cell-intrinsic mechanisms regulating MSC differentiation. $PPAR_{\gamma}$ signaling promotes MSCs differentiation down the adipogenic lineage. Mechanical strain induced by exercise has been observed to decrease $PPAR_{\gamma}$ expression in MSCs, favoring osteogenesis over adipogenesis *in vivo* (89) and *in vitro* (23, 29, 127, 133). $PPAR_{\gamma}$ expression is activated by long-chain fatty acids (126), suggesting that hyperlipidemia induced by high fat diet stimulates MSCs adipogenesis. Interestingly, these signals seem to be overridden by exercise in the presence of a $PPAR_{\gamma}$ agonist (139). Metabolic stress has been linked to changes within the bone marrow stromal tissues. Bone marrow stromal cells incubated in the serum of overweight individuals promoted adipogenic differentiation over osteoblastic differentiation (43). While the underlying mechanism remain undetermined, this does suggest that unknown circulating factors may be influencing MSC fate determination within the bone marrow MSCs.

Overall, obesity and exercise seem to elicit functionally opposite results within the bone marrow (Figure 2 see next page). Obesity skews MSCs towards adipocyte lineages, increases the accumulation of MAT, and creates a more pro-inflammatory phenotype similar to aging. These changes in the niche are associated with altered HSC differentiation that favors increased myeloid biased progenitors, increased HSPC cycling, and mobilization leading to exhaustion of primitive HSPC populations (11, 106, 135, 155). Exercise and physical activity decrease the accumulation of MAT even in the pro-adipogenic conditions, suggesting that exercise may be a

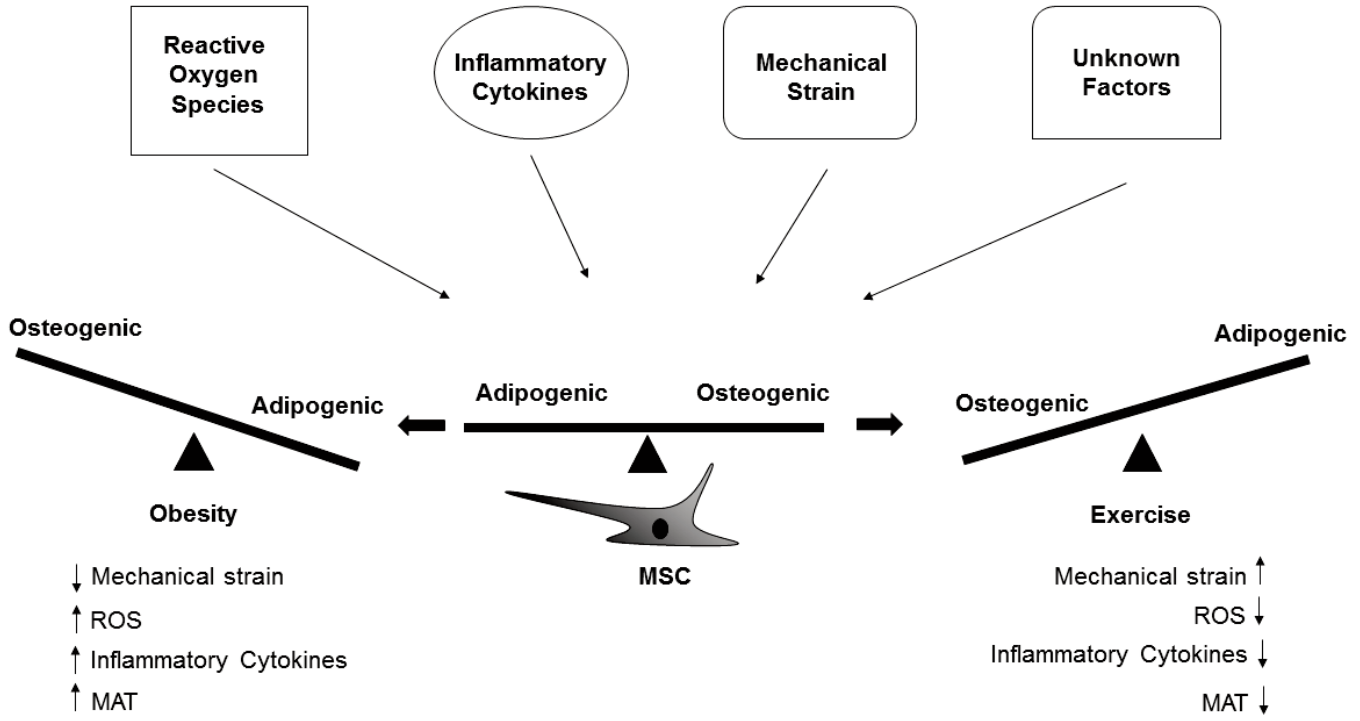

Figure 2. Factors influencing mesenchymal stromal cell differentiation (MSC). Systemic and bone marrow specific factors influence the differentiation of MSCs down adipogenic or osteogenic lineages. Obesity is associated with an increase in bone marrow adiposity likely due to increases of these factors. Exercise decreases marrow adipose tissue and increase bone marrow density, indicating that MSCs may be stimulated down an osteogenic lineage by decreasing reactive oxygen species and inflammatory cytokines while increasing mechanical strain within the bone marrow. Abbreviations: Mesenchymal stromal cell (MSC), Reactive oxygen species (ROS), and Marrow adipose tissue (MAT).

potent therapy to combat obesity induced changed to the bone marrow environment. However, further investigations are needed to fully delineate the multi-faceted responses of exercise in the context of the bone marrow stroma. Only a few studies have elucidated the impact of exercise on the HSPC populations (36, 38), none exist characterizing the impact on changes in MSCs, endothelial cells, or HSPC localization.

Conclusion and Future Directions

Obesity has a direct impact on the fate of the hematopoietic system (135), distressing normal immunological function (112) and phenotype (103). This dysfunction extends to HSPCs resulting in expansion of the less primitive progenitor cell pool, at the cost of self-renewal leading to premature exhaustion. The altered phenotype and function of HSPCs is likely due to modulations within the bone marrow niche. HSPC function is carefully regulated by niche localization, and disruption of cellular constituents can disrupt HSPC homeostasis. Although evidence does suggest that bone marrow niche disruption contributes to altered HSPC function in obesity, the systemic disruptions including elevated levels of pro-inflammatory cytokines associated with obesity, negates the ability to definitively characterize the primary suspect in altered HSPC function.

Chronic exercise directly combats the systemic effects of obesity by improving body composition (41), improving immune function (115), and decreasing chronic low grade inflammation (128). The pleiotropic responses of exercise extend to the bone marrow, inducing beneficial bone marrow remodeling and expanding the HSPC pool without compromising self-renewal. Although these preliminary data are promising, many questions remain unanswered pertaining to the mechanisms responsible for the effects of exercise on the bone marrow compartment and HSPC function. An in-depth analysis determining the extent to which exercise expands the whole HSPC pool or specific sub-populations still does not exist. In addition, although exercise has been identified to decrease overall bone marrow adiposity and alter the inflammatory status of the bone marrow; alterations in the individual cell populations making up distinct niches within the bone marrow still needs to be characterized. Furthermore, defining the molecular pathways by which exercise influences HSCPs, MSCs, and the bone marrow niche may highlight potential therapeutic pathways to combat obesity and hematological malignancies. Overall, the effects of exercise on the hematopoietic system need further characterization; however, exercise still remains a viable and feasible method of combatting obesity induced changes to the hematopoietic system.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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